REMARKS/ARGUMENTS

The claims in the accompanying continuation patent application recite terms such as "addition salt". A claim rejection under 35 U.S.C. § 112 ¶ 2 issued in the parent case regarding the same terms. For reasons such as those provided below, Applicants submit that these terms are not indefinite, and respectfully request that they not be the subject of such rejection.

The terms "addition salt" are widely used in the art, and their meaning is clear and definite to any person of ordinary skill in the art. These terms are typically used in the art without providing any definition or further characterization.

For example, the US PTO Classification Manual[1] recites as follows: "This subclass is intended under subclass 1. Compounds under Class 532, ... which contain nitrogen in a form other than as nitrogen in an inorganic ion of an addition salt, nitro, or nitroso." (Classification Definitions. Class 564, Organic Compounds – Part of the Class 532-570 Series, Subclass 1, p. 564-1, Dec. 2000 Edition). The same Manual further recites "[i]f amino nitrogen is present, the compound may additionally contain nitro, nitroso, or nitrogen in an inorganic ion of an addition salt." (Classification Definitions. Class 564, Organic Compounds – Part of the Class 532-570 Series, Subclass 1, note (4), p. 564-1, Dec. 2000 Edition).

Additional examples of classification rules that refer to addition salts include the following: "... wherein the claim includes a generic reference to salts, such as : ... 'or therapeutically useful acid addition salts thereof' ..., will have its original classification determined by ..." (Classification Definitions. Class 532, Organic Compounds – Part of the Class 532-570 Series, Section II.D, p. 532-3, Dec. 2000 Edition). *See also* Classification Definitions. Class 532, Organic Compounds – Part of the Class 532-570 Series, Section III, p. 532-6 (Dec. 2000 Edition) (referring to "any nitrogen in an organic compound other than a

[1] Copies of the relevant pages of the US PTO Classification Manual that correspond to the cites given herein are provided in Attachment "A" to this Preliminary Amendment.

nitrogen in an inorganic ion of an addition salt ....", in the glossary listing of the terms "amino nitrogen").

As further examples, the Classification Definitions also provide as follows: "Boron, silicon, or phosphorous containing active ingredient wherein the boron, silicon, or phosphorous is other than solely as part of an inorganic ion in an addition salt" (regarding subclass 153 in Classification Definitions. Class 504, p. 504-12 (Dec. 2000 Ed.)); "[p]hosphorous containing active ingredient wherein the phosphorous is other than solely as part of an inorganic ion in an addition salt" (regarding subclass 165 in Classification Definitions. Class 504, p. 504-14 (Dec. 2000 Ed.)); and "[p]hosphorous containing active ingredient wherein the phosphorous is other than solely as part of an inorganic ion in an addition salt" (regarding subclass 194 in Classification Definitions. Class 504, p. 504-17 (Dec. 2000 Ed.)).

Applicants submit that the terms "addition salt" and grammatically related terms must satisfy the threshold requirements of clarity and precision if the same terms are ordinarily used as such in the classification of inventions by the US PTO. In light of at least this usage, Applicants respectfully submit that the terms "addition salt" and grammatically related terms define subject matter with a reasonable degree of particularity and distinctness, and that these terms appraise one of ordinary skill in the art of their scope and consequently serve the notice function required by 35 U.S.C. § 112 ¶ 2.

The claims in the accompanying continuation patent application also recite terms such as "quaternary amine". A claim rejection under 35 U.S.C. § 112 ¶ 2 issued in the parent case regarding the same terms. For reasons such as those provided below, Applicants submit that these terms are not indefinite, and respectfully request that they not be the subject of such rejection.

The terms "quaternary amine" are widely used in the art, and their meaning is clear and definite to any person of ordinary skill in the art. These terms are typically used in the art without providing any definition or further characterization.

The terms "quaternary amine" are used in numerous areas of chemistry, including organic chemistry. These terms are commonly used[2] in enzyme terminology (*see, e.g.*, <http://www.biochem.ucl.ac.uk/bsm/enzymes/ec3/ec06/ec03/ec0032/>, reporting on enzymes that have been deposited in the Brookhaven Protein Data Bank, and particularly on "quaternary-amine-transporting ATPase"); solid-phase resins and ion exchange chromatography (*see, e.g.*, <www.sigma-aldrich.com>, referring to resins with quaternary amine functionality; S. Levin, "Ion Exchange Chromatography", Medtechnica, referring to various quaternary amines as exchangers; and "96 Well SPE Plates", United Chemical Technologies, Inc., referring to various quaternary amines as ion exchange sorbents); mass spectrometry (*see, e.g.*, F.J. Cox, A Dasgupta, and M.V. Johnston, "Matrix-assisted laser desorption/ionization mass spectrometry of amine functionalized polystyrenes", University of Delaware, Newark, Delaware, characterizing tertiary amine and quaternary amine functionalized polystyrenes); chemical analysis (*see, e.g.*, jtbaker.com Technical Library at <http://www.jtbaker.com/techlib/documents/ph-014.html>, referring to a quaternary amine as one of the products to be used in the extraction of vitamin B_{12} from multivitamin tablets); and receptor modulation (*see, e.g.*, M. Zhou, J.H. Morais-Cabral, S. Mann, and R. Mackinnon, "Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors", Nature, vol. 411, pp. 657-661 (2001), referring to quaternary amines as pore blockers and as competitors in the inhibition of K^+ current). Furthermore, a printout of a Medline Repository with references that use the terms "quaternary amine" and grammatically related terms, and their abstracts, are also provided herein. These sample works

[2] Copies of the citations provided herein as examples are given in Attachment "B" to this Preliminary Amendment.

illustrate the wide use of the terms "quaternary amine" and they also illustrate that these terms are commonly understood in chemistry.

Applicants submit that the terms "quaternary amine" and grammatically related terms must satisfy the threshold requirements of clarity and precision if the same terms are ordinarily used within a broad spectrum of the chemical literature. In light of at least this usage, Applicants respectfully submit that the terms "quaternary amine" and grammatically related terms define subject matter with a reasonable degree of particularity and distinctness, and that these terms appraise one of ordinary skill in the art of their scope and consequently serve the notice function required by 35 U.S.C. § 112 ¶ 2.

Applicants respectfully request a favorably consideration of the accompanying continuation patent application.

Respectfully submitted,

By:____________________________
Jesús Juanós i Timoneda, PhD
Reg. No. 43,332

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New Brunswick, NJ 08933-7003
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Dated: August 5, 2003

U.S. Express Mail No. EF195551352US Docket No. JAB1425CON1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Bart DE CORTE, *et al.*
Serial No.	:	To be assigned
Filed	:	August 5, 2003
Title	:	HIV REPLICATION INHIBITING PYRIMIDINES
Art Unit	:	1624
Examiner	:	Venkataraman Balasubramanian (parent application)
Confirmation No.:		To be assigned

ATTACHMENT "A"

TO

PRELIMINARY AMENDMENT "A"

Copies of the following items are provided in pages 7-12 of this Attachment:

- Classification Definitions. Class 564, Organic Compounds – Part of the Class 532-570 Series, Subclass 1, p. 564-1, Dec. 2000 Edition;
- Classification Definitions. Class 564, Organic Compounds – Part of the Class 532-570 Series, Subclass 1, note (4), p. 564-1, Dec. 2000 Edition;
- Classification Definitions. Class 532, Organic Compounds – Part of the Class 532-570 Series, Section II.D, p. 532-3, Dec. 2000 Edition;
- Classification Definitions. Class 532, Organic Compounds – Part of the Class 532-570 Series, Section III, p. 532-6, Dec. 2000 Edition;
- Classification Definitions. Class 504, p. 504-12, Dec. 2000 Edition;
- Classification Definitions. Class 504, p. 504-14 Dec. 2000 Edition; and
- Classification Definitions. Class 504, p. 504-17 Dec. 2000 Edition.

CLASS 564, ORGANIC COMPOUNDS -- PART OF THE CLASS 532-570 SERIES

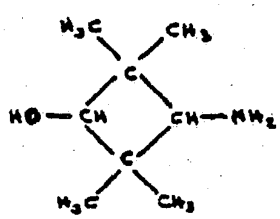
SUBCLASSES

1 This subclass is indented under subclass 1. Compounds under Class 532, ... which contain nitrogen in a form other than as nitrogen in an inorganic ion of an addition salt, nitro, or nitroso.

(1) Note. This group of compounds includes for example, ureas, thioureas, amides, amidines, azines, hydrazones, carbodiimides, oximes, hydroxylamines, and amines, inter alia, as well as their inorganic acid salts.

(2) Note. This subclass is residual for alicyclic amines not specifically provided for below.

(3) Note. This subclass contains, for example:



(4) Note. If amino nitrogen is present, the compound may additionally contain nitro, nitroso, or nitrogen in an inorganic ion of an addition salt.

(5) Note. Component parts of an "adduct" will be considered to be attached to each other ionically, except if it is clear that the mode of attachment is nonionic.

SEE OR SEARCH CLASS:

588, Hazardous or Toxic Waste Destruction or Containment, subclasses 206 through 225 for the destruction of organic hazardous or toxic waste containing halogen, sulfur, oxygen, nitrogen, phosphorus, or metals.

1.5 This subclass is indented under subclass 1. Compounds wherein urea, per se, of thiourea, per se, forms an adduct or inclusion compound with an organic compound.

(1) Note. by adduct or inclusion compound is meant a type of complex in which the urea or thiourea is bound with another suitable chemical without changing the chemical character of either the urea-thiourea or of the other chemical; the respective molecules will be unaltered in their chemical nature and the individual compounds may readily be constituted and isolated.

(2) Note. An example of a compound provided for herein is the adduct of urea and an alkane.

2 This subclass is indented under subclass 1. Products wherein the amino nitrogen containing compound is mixed with a preserving agent whose sole function is to prevent physical or chemical change.

3 This subclass is indented under subclass 2. Products wherein the compound stabilized or preserved contains the grouping below, wherein X is O or S. NN

4 This subclass is indented under subclass 2. Products wherein the compound stabilized or preserved is a carboxamide containing the grouping RN

5 This subclass is indented under subclass 2. Products wherein the compound being preserved contains a benzene ring.

6 This subclass is indented under subclass 5. Products wherein the preserving or stabilizing agent is inorganic.

7 This subclass is indented under subclass 5. Products wherein the preserving or stabilizing agent contains sulfur or a phenolic group.

8 This subclass is indented under subclass 1. Compounds which contain boron.

(1) Note. This subclass contains boron containing complexes, adducts, and salts.

Purification or recovery steps would not effect classification in the above illustrations. Classification is determined by the controlling synthesis step.

D. SPECIAL RULES FOR CLASSIFYING SALTS

The rule to be utilized in classifying and cross referencing generic claims to salts in this series of Classes is clarified here. This rule applies only to salts and is not to be considered analogous to nor does it apply to other types of claimed disclosure.

A patent wherein the controlling claim is to a "compound" (e.g., acid or base) and wherein the claim includes a generic reference to salts, such as: "and the pharmacologically acceptable salt thereof", "or therapeutically useful acid addition salts thereof", "and non-toxic heterocyclic amine salts thereof", etc., will have its original classification determined by the "compound" without regard to the generic reference to the salts thereof. A patent in which the generic reference to salts is in a separate claim which is dependent on a claim to the "compound" is considered equivalent and will also have its original classification determined by the "compound" without regard to the generic reference to the salts thereof.

Cross-referencing of such a patent for a salt is mandatory only when it is clear that the specific salt was actually made as evidenced by: (a) a "working example" of a specific salt, (b) a property of a specific salt, such as its melting point, infrared scan, nuclear magnetic resonance, etc., or (c) an example of using a specific salt, such as in the treatment of animal life. Other cross referencing of salts, such as those which are part of a list in the disclosure, is optional and should be made only when clearly useful.

When a specific salt is set forth in a claim, the entire compound will be considered in determining the original classification, i.e., the original will be placed on the basis of the first appearing subclass providing for the acid, base, or salt. A specific salt is considered to be set forth in a claim when the structure of the salt forming moiety is clear from the claim or when the claim specifies that a heavy metal or a specific hetero ring (e.g., "and substituted morpholine containing salts thereof", etc.) is present in the salt forming moiety.

Other claims are treated the same as the controlling claim when considering where to cross reference, i.e., any generic reference to salts is disregarded as explained above.

E. CLASSIFYING COMPOUNDS OF UNKNOWN STRUCTURE WITHIN THIS SERIES OR CLASSES.

Classifying compounds of unknown structure in this Series of Classes is accomplished by considering two possible methods for classifying them and employing the one which results in the highest classification in the Series. The two methods are: 1. Classify according to an element or group of elements known to be part of the compound. 2. Classify based on an organic reactant utilized to make the compound.

When considering the first method, compounds are classified based on any partial structure of the compound which is known or which can be found by looking up a named compound in published sources. For example, if a specific alkaloid is named in a patent and if the structure or partial structure for that alkaloid can be found, the patent is classified according to that structure or partial structure. Patents claiming unnamed alkaloids in general have been classified in Class 546, subclass 1 on the assumption that alkaloids usually include a ring consisting of one nitrogen and five carbons.

Another situation involving unknown structures involves "oxidized hydrocarbon" in which there is no disclosure as to the structure of the products. These are placed in Class 568 in an indent under "oxygen containing". All that is known about them are the elements they contain. However, sulfurized carbohydrates of unknown structure are placed with carbohydrates based on the organic starting material. The "sulfur containing" subclasses are lower in the Series than carbohydrates in Class 568. Sulfurized nitro containing organic compounds are classified with "sulfur containing" because that is higher in the Class 568 schedule than "nitro containing".

Compounds which are disclosed as carbohydrates, proteins, lignins, starch, etc., and which are provided for according to titles of the Series are considered known structures, even though the exact structure isn't set forth in the patent. They will be classified as known compounds and will not be treated as compounds of unknown structure or undetermined constitution.

F. LINES BETWEEN COMPOSITION CLASSES AND THIS SERIES OF CLASSES

In general, the 532-570 Series of Classes takes mixtures of organic compounds only if the mixtures:

(A) result from a chemical process or synthesis wherein

tains a carbon-to-carbon double bond and is represented by the formula $-C_nH_{2n-1}$.

ALKENYLENE

This term denotes an acyclic carbon chain which contains a carbon-to-carbon double bond and is represented by the formula $-(C_nH_{2n-2})-$.

ALKYL

This term denotes an acyclic carbon or a saturated acyclic carbon chain represented by the formula $-C_nH_{2n+1}$.

ALKYLENE

This term denotes an acyclic carbon or a saturated acyclic carbon chain represented by the formula C_nH_{2n-}.

ALKYNYL

This term denotes an acyclic carbon chain which contains a carbon-to-carbon triple bond and is represented by the formula $-(C_nH_{2n-3})-$.

ALKYNLENE

This term denotes an acyclic carbon chain which contains a carbon-to-carbon triple bond and is represented by the formula $-(C_nH_{2n-4})$.

AMINO NITROGEN

Denotes any nitrogen in an organic compound other than a nitrogen in an inorganic ion of an addition salt, a nitro ($-NO_2$) or nitroso ($-NO$). Component parts of an "adduct" will be considered to be attached to each other ionically except if it is clear that the mode of attachment is nonionic.

ARYL RING OR RING SYSTEM

This term denotes a benzene ring or a polycyclo carbocyclic ring system having a benzene ring as one of the cyclos.

ATTACHED DIRECTLY OR BONDED DIRECTLY

These terms are used to show that specified moieties are connected by bonds only.

ATTACHED INDIRECTLY

This term denotes that at least one atom, as well as bonds, connects specified moieties.

BENZENE RING

This term includes, in all cases except where there are explicit limitations to the contrary, substituted benzene rings, including substitution in the form of an additional fused or bridged ring or ring system.

Thus, for example, if a subclass reads: "Benzene ring bonded directly to the five-membered hetero ring", the moiety bonded directly to the hetero ring may be phenyl, chlorophenyl, dinitrophenyl, naphthyl, etc. All that is necessary to satisfy the terminology of the subclass is that a substituted or unsubstituted benzene ring be bonded directly to the hetero ring.

CARBOCYCLIC

This term denotes a ring or ring system where all ring members are carbons.

CHAIN

This term denotes a plurality of atoms which connect specified groups or atoms. The atoms of the chain must be nonionically attached to each other and to the specified groups or atoms. If the chain may not include any ring members it will be designated as acyclic. When the chain may include ring members the title will state that the chain may include a ring. The chain ends where it attaches to the specified groups or atoms and does not include any part of them. The chain may have substituents but the substituents are not part of the chain.

CLATHRATES AND INTERCALATES (INCLUSION COMPOUNDS)

Clathrates and intercalates (inclusion compounds), per se, are classified hierarchically and subject to the limitations set forth in the compound (element) classes based both on the encapsulant and encapsulate. For example, a clathrate of urea and hydrogen peroxide is classified in Class 564, subclass 32, urea and an organic compound in Class 564, subclass 1.5, dextran and iodine in Class 536, subclass 112, etc. Where a patent does not state that a material is either a clathrate or an intercalate, the assumption is made that the material is either a coated or encapsulated product classified in Class 428, subclasses 402+.

CONTAINING

210, Liquid Purification or Separation, subclasses 601+, especially 636, 753+, and 764 processes for destroying micro-organisms in a liquid medium which are more than the mere addition of a compound or composition to said liquid.

435, Chemistry: Molecular Biology and Microbiology, particularly subclasses 257.1+ for subject matter directed to a composition having utility as an algal culture medium (i.e., media for maintenance, growth, production, etc.) or a technique for preparing or using the same.

151 **Inorganic active ingredient containing:**
This subclass is indented under subclass 150. Compositions wherein the active aquatic plant regulating agent is an element or an inorganic compound.

152 **Heavy metal or aluminum containing active ingredient:**
This subclass is indented under subclass 150. Compositions wherein the active aquatic plant regulating agent is an organic compound which contains aluminum or a metal having a specific gravity greater than four.

(1) Note. Arsenic is considered a heavy metal.

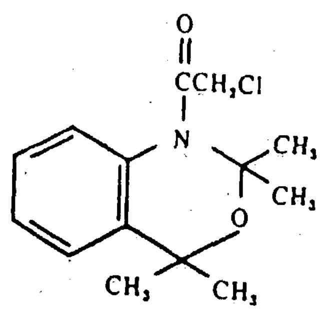
153 **Boron, silicon, or phosphorus containing active ingredient wherein the boron, silicon, or phosphorus is other than solely as part of an inorganic ion in an addition salt:**
This subclass is indented under subclass 150. Compositions in which the active aquatic plant regulating agent contains an organic compound wherein boron, silicon, or phosphorus is attached directly or indirectly to carbon by nonionic bonding.

(1) Note. Inorganic boron, silicon, or phosphorus salts of the active aquatic plant regulating agent are excluded herefrom and classified with the active organic moiety.

154 **Hetero ring containing active ingredient:**
This subclass is indented under subclass 150. Compositions wherein the active aquatic plant regulating agent contains a hetero ring.

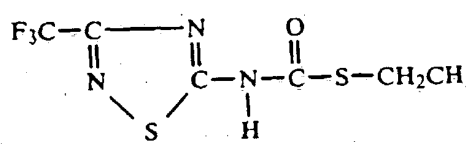
155 **Hetero ring includes nitrogen:**
This subclass is indented under subclass 154. Compositions wherein the hetero ring contains nitrogen as a ring member.

(1) Note. An example of a compound provided for herein is:



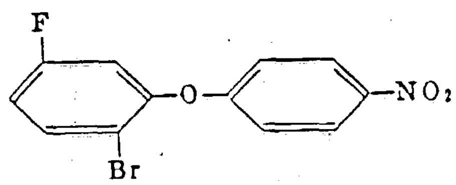
156 **Hetero ring is five-membered (e.g., thiadiazoles, etc.):**
This subclass is indented under subclass 155. Compositions wherein the hetero ring is five-membered.

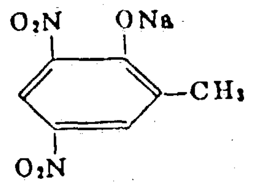
(1) Note. An example of a compound provided for herein is:



157 **Active ingredient contains -C(=X)X-, wherein the X's are the same or diverse chalcogens (e.g., carbamates, thiocarbamates, carboxylic acids, etc.):**
This subclass is indented under subclass 150. Compositions in which the active aquatic plant regulating agent contains a -C(=X)X- group, wherein the X's are the same or diverse chalcogens (i.e., oxygen, sulfur, selenium, or tellurium).

(1) Note. An example of a compound provided for herein is:





162 **Abscission agent, defoliant, or dessicant:**
This subclass is indented under subclass 116.1. Compositions which are designed or intended for facilitating or causing fruit, blossom, or leaf drop, or for desiccating a living plant, e.g., premature drying.

163 **Inorganic active ingredient containing:**
This subclass is indented under subclass 162. Compositions wherein the active abscission, defoliant, or desiccant agent is an element or an inorganic compound.

164 **Boron, silicon, heavy metal, or aluminum containing active ingredient:**
This subclass is indented under subclass 162. Compositions wherein the active abscission, defoliant, or desiccant agent is an organic compound which contains boron, silicon, aluminum, or a metal having a specific gravity greater than four.

(1) Note. Arsenic is considered a heavy metal.

165 **Phosphorus containing active ingredient wherein the phosphorus is other than solely as part of an inorganic ion in an addition salt:**
This subclass is indented under subclass 162. Compositions in which the active abscission, defoliant, or desiccant agent contains an organic compound wherein phosphorus is attached directly or indirectly to carbon by nonionic bonding.

(1) Note. Inorganic phosphorus salts of the active abscission, defoliant, or desiccant agent are excluded herefrom and classified with the active organic moiety.

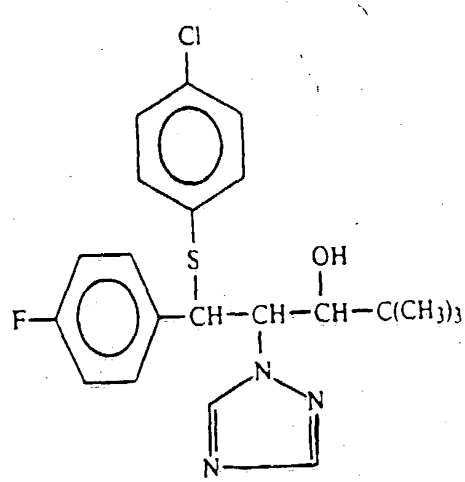
166 **Hetero ring containing active ingredient:**
This subclass is indented under subclass 162. Compositions wherein the active abscission, defoliant, or desiccant agent contains a hetero ring.

167 **Hetero ring is six-membered including nitrogen:**
This subclass is indented under subclass 166. Compositions wherein the hetero ring is six-membered and has nitrogen as a ring member.

168 **Plural ring nitrogens in the hetero ring:**
This subclass is indented under subclass 167. Compositions wherein the hetero ring contains at least two ring nitrogens.

169 **Hetero ring is five-membered having two or more ring hetero atoms of which at least one is nitrogen:**
This subclass is indented under subclass 166. Compositions in which the hetero ring is five-membered and has two or more hetero atoms as ring members, at least one of which is nitrogen.

(1) Note. An example of a compound provided for herein is:



188 Inorganic active ingredient is elemental nitrogen, elemental sulfur, or is a compound of nitrogen or sulfur:
This subclass is indented under subclass 116.1. Compositions wherein an inorganic active ingredient is elemental nitrogen, elemental sulfur, or is a compound of nitrogen or sulfur.

189 Organic active compound containing:
This subclass is indented under subclass 116.1. Compositions containing an organic compound as an active plant growth regulating agent.

(1) Note. Included herein are organic substances of unknown constitution.

190 Heavy metal or aluminum containing:
This subclass is indented under subclass 189. Compositions wherein the organic active compound contains aluminum or a metal having a specific gravity greater than four.

(1) Note. Arsenic is considered a heavy metal.

191 Hetero ring containing:
This subclass is indented under subclass 190. Compositions which contain a hetero ring.

192 Group IV or V heavy metal (e.g., Sn, As, Ti, etc.):
This subclass is indented under subclass 190. Compositions wherein the heavy metal is germanium, tin, lead, titanium, zirconium, hafnium, arsenic, antimony, bismuth, vanadium, niobium, or tantalum.

193 Boron or silicon containing:
This subclass is indented under subclass 189. Compositions wherein the organic active compound contains boron or silicon.

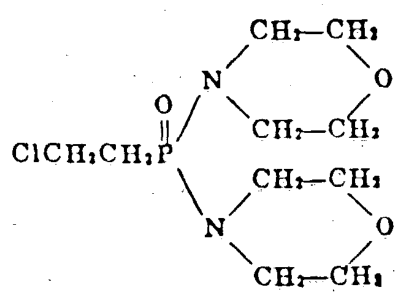
194 Phosphorus containing wherein the phosphorus is other than solely as part of an inorganic ion in an addition salt:
This subclass is indented under subclass 189. Compositions wherein the organic active compound contains phosphorus attached directly or indirectly to carbon by nonionic bonding.

(1) Note. Salts of the organic active compound with an inorganic phosphorus compound are classified with the active organic moiety.

195 Hetero ring containing:
This subclass is indented under subclass 194. Compositions which contain a hetero ring.

196 Ring chalcogen in the hetero ring (e.g., morpholines, etc.):
This subclass is indented under subclass 195. Compositions wherein the hetero ring has chalcogen as a ring member.

(1) Note. An example of a compound provided for herein is:



197 Plural ring nitrogens in the hetero ring:
This subclass is indented under subclass 195. Compositions wherein the hetero ring contains at least two ring nitrogens.

198 Having -C(=X)-, wherein X is chalcogen, bonded directly to the phosphorus:
This subclass is indented under subclass 194. Compositions wherein the phosphorus is bonded directly to a -C(=X)- group, wherein X is chalcogen (i.e., oxygen, sulfur, selenium, or tellurium).

199 Nitrogen bonded directly to the phosphorus:
This subclass is indented under subclass 194. Compositions wherein the phosphorus is bonded directly to nitrogen.

200 Plural nitrogens bonded directly to the phosphorus:
This subclass is indented under subclass 199. Compositions wherein phosphorus is bonded directly to two or more nitrogens.

U.S. Express Mail No. EF195551352US Docket No. JAB1425CON1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Bart DE CORTE, *et al.*
Serial No.	:	To be assigned
Filed	:	August 5, 2003
Title	:	HIV REPLICATION INHIBITING PYRIMIDINES
Art Unit	:	1624
Examiner	:	Venkataraman Balasubramanian (parent application)
Confirmation No.:		To be assigned

ATTACHMENT "B"

TO

PRELIMINARY AMENDMENT "A"

Copies of the following citations are provided in pages 14-49 of this Attachment:

- <http://www.biochem.ucl.ac.uk/bsm/enzymes/ec3/ec06/ec03/ec0032/>;
- <www.sigma-aldrich.com>;
- S. Levin, "Ion Exchange Chromatography", Medtechnica;
- "96 Well SPE Plates", United Chemical Technologies, Inc.;
- F.J. Cox, A Dasgupta, and M.V. Johnston, "Matrix-assisted laser desorption/ionization mass spectrometry of amine functionalized polystyrenes", University of Delaware, Newark, Delaware;
- jtbaker.com Technical Library at <http://www.jtbaker.com/techlib/documents/ph-014.html>;
- M. Zhou, J.H. Morais-Cabral, S. Mann, and R. Mackinnon, "Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors", Nature, vol. 411, pp. 657-661 (2001);
- printout of a Medline Repository with 11 references and their corresponding abstracts.

PDBsum Enzymes E.C.3.6.3.32

E.C.3.-.-.- Hydrolases.

E.C.3.6.-.- Acting on acid anhydrides.

E.C.3.6.3.- Acting on acid anhydrides; catalyzing transmembrane movement

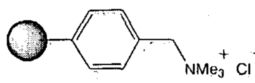
E.C.3.6.3.32 Quaternary-amine-transporting ATPase.

Reaction: *Atp + H(2)O + quaternary amine(Out) = adp + phosphate + quaternary amine(In).*
Comments: *ABC-type (ATP-binding cassette-type) ATPase, characterised by the presence of two similar ATP-binding domains. A bacterial enzyme that imports betaine and glycine.*

Links to other enzyme databases: ExPaSy KEGG WIT BRENDA

There are no PDB entries in enzyme class E.C.3.6.3.32

PDBsum Enzymes E.C.3.6.3.32

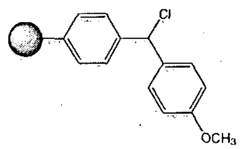


51,797-6

Poly(styrene-co-divinylbenzene), quaternary amine functionality, chloride form

100g
500g

Specifications: 1% DVB, 15-50 mesh, 3.5-4.5 mmol Cl/g



53,469-2

4-Methoxybenzhydryl chloride, polymer-bound

5g
25g
100g

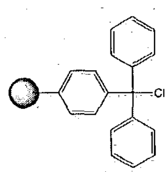
Synonym(s): MAMP-Cl resin; Merrifield, α-methoxyphenyl resin

Specifications: 1% DVB, 50-90 mesh, *ca.* 1.0 mmol Cl/g

MAMP resin is useful for the on-resin synthesis and mild acidolytic cleavage of compounds containing secondary amide functionality.

Brown, D.S. et al. *Tetrahedron Lett.* **1998**, *39*, 8533.

TRITYL RESINS



53,348-3

Trityl chloride, polymer-bound

1g
5g
25g

Specifications: 1% DVB, 100-200 mesh, 1.0-1.8 mmol Cl/g

This resin has been used to attach alcohols,[1-5] amines,[6-9] and acids.[7] It has also been used in the Pauson-Khand reactions of norbonene-derived substrates.[10]

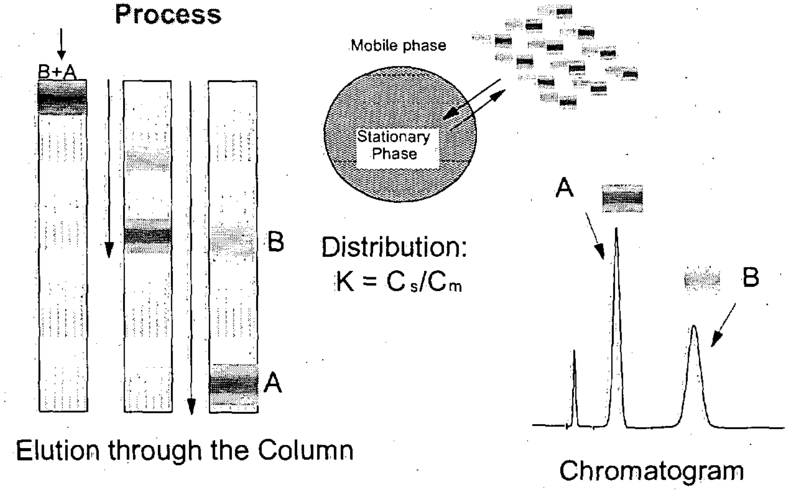
Decarboxlyation-based traceless linking with aroyl acrylic acids has been demonstrated. The acids are esterified to the resin, followed by Michael-type addition of indolines. Upon cleavage, the products are decarboxylated in a traceless manner.[11]

(1) Chen, C. et al. *J. Am. Chem Soc.* **1994**, *116*, 2661. (2) Barlos, K. et al. *Tetrahedron Lett.* **1989**, *30*, 3943. (3) Lenzoff, C.C. et al. *ibid.* **1982**, *23*, 3023. (4) Chen, C. et al. *Tetrahedron* **1997**, *53*, 6595. (5) Gennari, C. et al. *ibid.* **1998**, *54*, 14999. (6) Bauer, U. et al. *Tetrahedron Lett.* **1997**, *38*, 7233. (7) Matthews, D.P. et al. *J. Comb. Chem.* **2000**, *2*, 19. (8) Barco, A. et al. *ibid.* **2000**, *2*, 337. (9) Guan, Y. et al. *ibid.* **2000**, *2*, 297. (10) Spitzer, J.L. et al. *Tetrahedron* **1997**, *53*, 6791. (11) Garibay, P. et al. *Tetrahedron Lett.* **1998**, *39*, 2207.

Ion Exchange
Chromatography

Dr. Shulamit Levin
Medtechnica

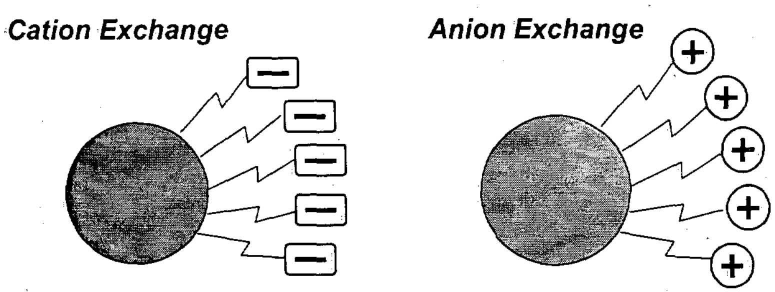
Chromatographic Process



Distribution:
$K = C_s/C_m$

Ion Exchange Theory

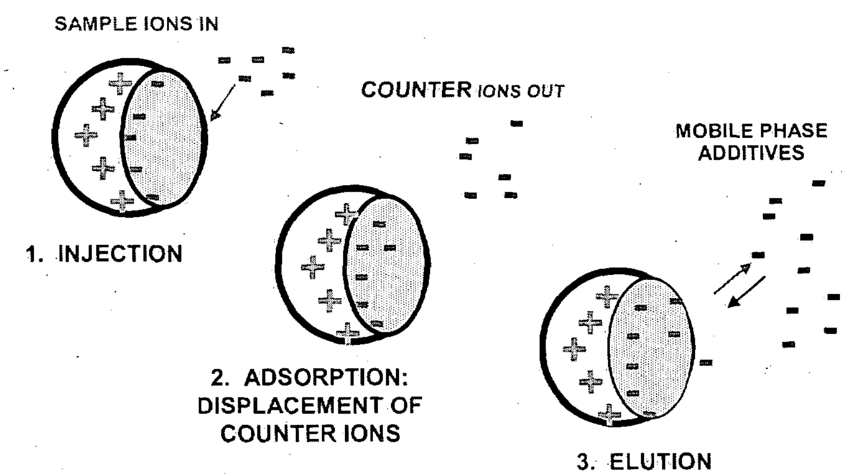
Cation Exchange vs Anion Exchange



Cation exchange columns have a negative charge to attract cations.
Anion exchange columns have a positive charge to attract anions

ION EXCHANGE

INSIDE A PORE IN THE STATIONARY PHASE



ION EXCHANGER

ANION EXCHANGE

$$\overline{B^+Y^-} + X^- \underset{}{\overset{K_{XY}}{\rightleftarrows}} \overline{B^+X^-} + Y^-$$

FUNCTIONAL

CATION EXCHANGE

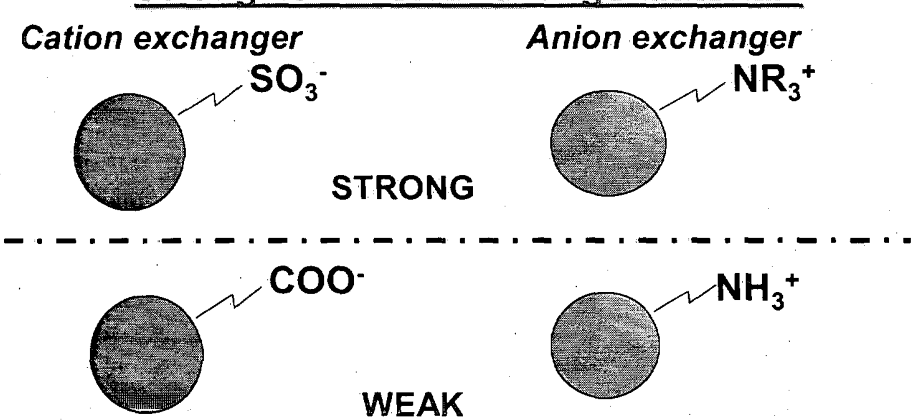
$$\overline{A^-M^+} + NH^+ \underset{}{\overset{K_{M\,NH}}{\rightleftarrows}} \overline{A^-NH^+} + M^+$$

— IMMOBILIZED ON THE STATIONARY PHASE

-17-

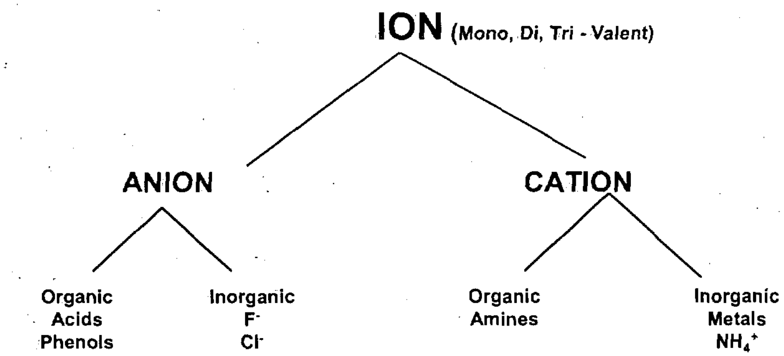
Ion Exchange Theory

Strong vs. Weak Exchange Materials



Strong Exchangers stay ionized as pH varies between 2 and 12.
Weak exchangers can lose ionization as a functionof pH.

Analysis of Ions - Ion Chromatography



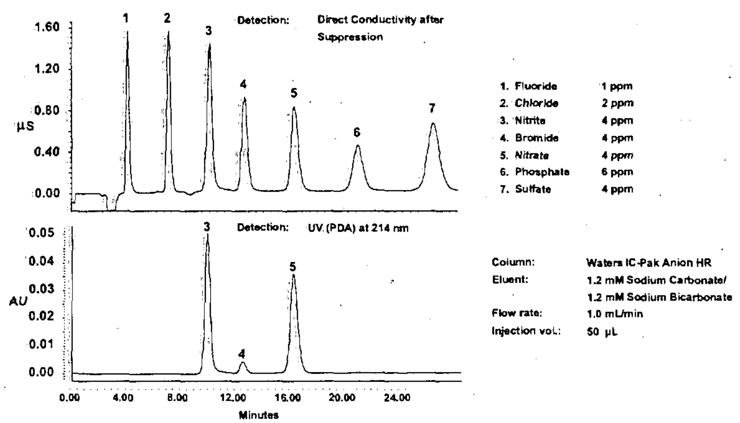
Ions can be characterized as:
organic or inorganic, anion or cation, mono or polyvalent.

Ion Exchange - Bonded Functionalities

	Cation	Anion
WEAK	∿∿ $COO^- Na^+$ Carboxylic Acid	∿∿ $N^+(R)(H)$-R Cl^- Primary, Secondary or Tertiary Amine
STRONG	∿∿ $SO_3^- Na^+$ Sulfonic Acid	∿∿ $N^+(R)(R)$-R Cl^- Quaternary Amine

Typical chemical functionalities used for commercial exchangers.

Conductivity and PDA Detectors in Series



Functional groups in Solutes

CATION EXCHANGERS		ANION EXCHANGERS	
TYPE	FUNCTIONAL GROUP	TYPE	FUNCTIONAL GROUP
Sulfonic acid	$-SO_3^- H^+$	Quaternary amine	$-N(CH_3)_3^+ OH^-$
Carboxylic acid	$-COO^- H^+$	Quaternary amine	$-N(CH_3)_2(EtOH)^+$
Phosphonic acid	$PO_3^- H^+$	Tertiary amine	$-NH(CH_3)_2^+ OH^-$
phosphinic acid	$HPO_2^- H^+$	Secondary amine	$-NH_2(CH_3)_2^+ OH^-$
Phenolic	$-O^- H^+$	Primary amine	$-NH_3^+ OH^-$
Arsonic	$-HAsO_3^- H^+$		
Selenonic	$-SeO_3^- H^+$		

Columns' Matrices

- Silica-Based
- Polymer-based ion-exchangers
- Hydrous Oxide

$$=M\text{-}O\text{-}H \rightleftarrows =M\text{-}O^- + H^+$$
$$=M\text{-}O\text{-}H \rightleftarrows =M^+ + OH^-$$

Ion Exchange Theory

Packing Supports

Resin **Silica-Based**

- **Capacity**
- **Swelling**
- **Mass Transfer**
- **Size Separation**
- **Reverse Phase**
- **Efficiency**
- **pH Range**
- **Equilibration**
- **Literature**

Both resin and silica based ion exchangers have advantages and disadvantages which are summarized here.

-18-

ION EXCHANGE

ANIONS

RETENTION & ELUTION STRENGTH

$F^- , OH^- > OAc^- > H_2PO_4^- > HCO_3^- > Cl^- > NO_2^- >$

$HSO_3^- > CN^- > Br^- > NO_3^- > I^-$

$A^{---} > A^{--} > A^-$

Properties of Mobile phases

- Compatibility with the detection mode - Suppressed or Non-suppressed.
- Nature of the competing ion
- Concentration of the competing ion
- Mobile phase's pH
- Buffering capacity of the mobile phase
- Ability to complex the ionic sample components
- Organic modifiers

ION EXCHANGE

CATIONS

RETENTION & ELUTION STRENGTH

MONO-VALENT

$Li^+ > H^+ > Na^+ > NH_4^+ > K^+ > Rb^+ > Cs^+ > Ag^+$

DI-VALENT

$Be^{++} > Mn^{++} Mg^{++} Zn^{++} > Co^{++} > M^{++} > Ca^{++} > Sr^{++}$

transition metals

TRI-VALENT

$Al^{+++} > M^{+++} > Ce^{+++}$

Transition metals

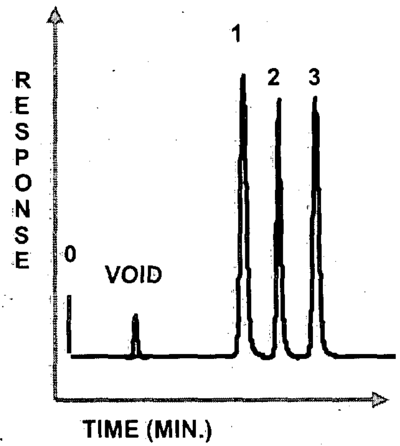
Ion capacity

The number of functional groups per unit weight of the stationary phase.

A typical ion-exchange capacity in IC is 10-100 mequiv/g.

-19-

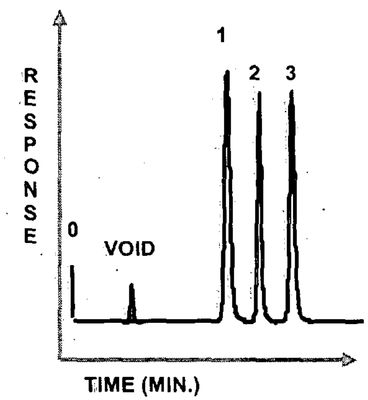
ELUTION ORDER IN ANION EXCHANGE



DENSITY OF CHARGE

1	2	3
OAc^-	OH^-	F^-
1	2	3
Cl^-	NO_3^-	NO_2^-
1	2	3
NO_3^-	SO_4^{2-}	SO_3^{2-}

ELUTION ORDER IN CATION EXCHANGE



DENSITY OF CHARGE

1	2	3
K^+	Na^+	Li^+
1	2	3
Ag^+	Zn^{++}	Al^{+++}

IONIZATION and RETENTION

WEAK ACIDS

$$HA \rightleftharpoons H^+ + A^-$$

pKa~ 4-5

At pH >4-5 the main species is A^-

WEAK BASES

$$HB^+ \rightleftharpoons H^+ + B$$

pKa ~ 7-8

At pH < 7-8 the main species is BH^+

The Equilibrium Constant

$$HAc \underset{k^2}{\overset{k^1}{\longleftrightarrow}} H^+ + Ac^- \qquad K_a = \frac{(H^+)\,(Ac)}{(HAc)}$$

pH and pK$_a$

$$(H^+) = K_a \frac{(HAc)}{(Ac)} \qquad pH = pK_a - \log \frac{(HAc)}{(Ac^-)}$$

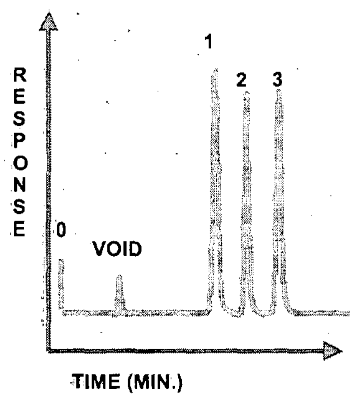
A general understanding of ionization constants, pH, and pK_a are useful in understanding ion exchange and buffer phenomena.

-20-

ELUTION ORDER IN ION EXCHANGE

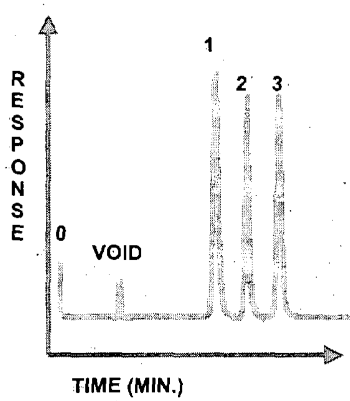
ANION EXCHANGE

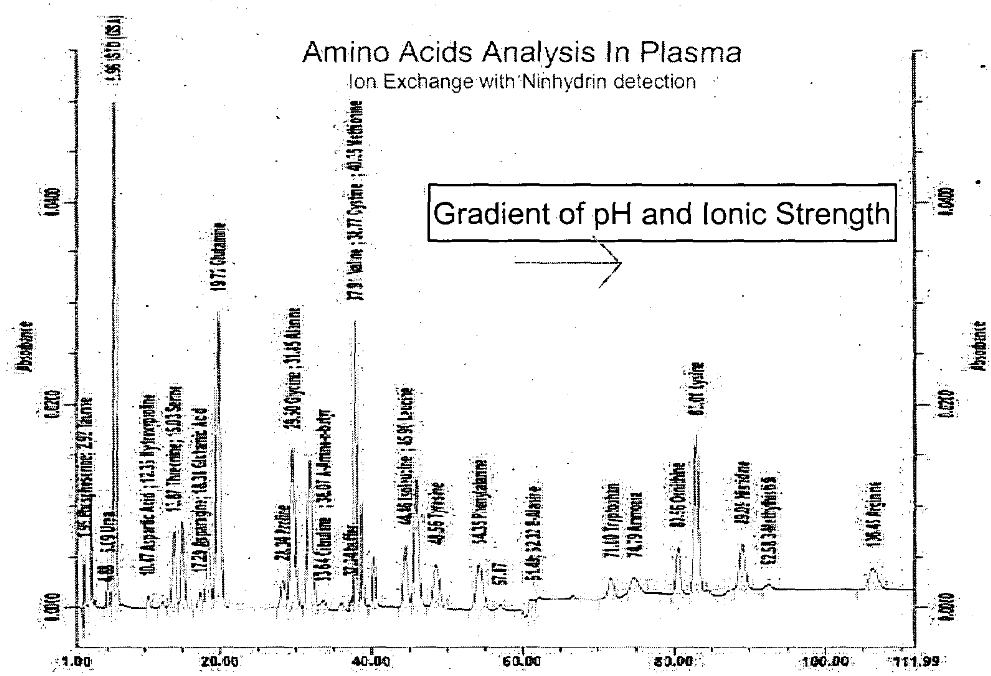
STRONGER ACID



CATION EXCHANGE

STRONGER BASE





Dr. Shulamit Levin, Analytical Consulting, Medtechnica

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*Now our sorbents are available in 96 Well Plates format!**

United Chemical Technologies, Inc. is the leader in the development of SPE products since 1984. This expertise is based upon a comprehensive knowledge of silica based sorbent manufacture which results in reproducible products of the highest quality.

- Over 35 different sorbent chemistries
- Method development formats
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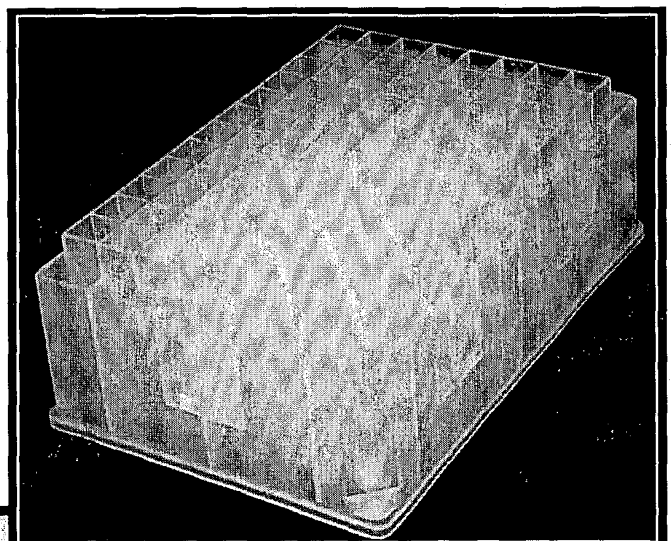
- Satisfaction guaranteed



IDEAL FOR ALL SPE APPLICATIONS AND HIGH THROUGH PUT SCREENING

- Up to a 2ml sample volume per well
- Robotic and Liquid Handling Compatibility: Advanced Chemtech, Beckman, Bodan, Gilson, Hamilton, Packard, Sagian, Tecan, Tomtec, Zinser, Zymark

Both 96 Well Plates are made from solvent resistant, low extractable polypropylene. Standard frits are polyethylene with 20µ pores. Different pore size or frit material is available upon request.

*A 96 well plate compatible with the Hydra® liquid handling system is also available.

Sorbents

Hydrophobic

SORBENT	STRUCTURE
C2 ethyl	$SiCH_2CH_3$
C3 propyl	$Si(CH_2)_2CH_3$
C4 n-butyl	$Si(CH_2)_3CH_3$
iC4 isobutyl	$SiCH_2CH(CH_3)_2$
tC4 tertiary butyl	$SiC(CH_3)_3$
C5 pentyl	$Si(CH_2)_4CH_3$
C6 hexyl	$Si(CH_2)_5CH_3$
C7 heptyl	$Si(CH_2)_6CH_3$
C8 octyl	$Si(CH_2)_7CH_3$
C10 decyl	$Si(CH_2)_9CH_3$
C12 dodecyl	$Si(CH_2)_{11}CH_3$
C18 octadecyl	$Si(CH_2)_{17}CH_3$
C20 eicosyl	$Si(CH_2)_{19}CH_3$
C30 tricontyl	$Si(CH_2)_{29}CH_3$
Cyclohexyl	Si-⬡
Phenyl	Si-⌬

Hydrophilic

SORBENT	STRUCTURE
Silica	SiOH
Diol	$Si(CH_2)_3OCH_2CHOHCH_2OH$
Cyanopropyl	$Si(CH2)_3CN$
Florisil PR®	
Alumina-Acid	
Alumina-Neutral	
Alumina-Base	

"Over 35 Different Phases"
Particle size 40-60 µm,
Pore size 60Å

Ion Exchange

SORBENT	STRUCTURE	pKa
Anion		
Aminopropyl (1° amine)	$Si(CH_2)_3NH_3^+$	9.8
n-2 aminoethyl (2° amine)	$Si(CH_2)_3NH_2^+(CH_2)_2NH_3^+$	10.1, 10.9
Diethylamino (3° amine)	$Si(CH_2)_3NH^+(CH_2CH_3)_2$	10.6
Quaternary Amine (4° amine)	$Si(CH_2)_3N^+(CH_3)_3$	always charged
• Available in alternative weaker counter ion; (CAQAX with $CH_3CO_2^-$ counter ion or CHQAX with OH^- counter ion)		
*** SAX (DVB / Styrene)		
Cation		
Carboxylic Acid	$SiCH_2COOH$	4.8
Propylsulfonic Acid	$Si(CH_2)_3SO_3H$	<1
Benzenesulfonic Acid	$Si(CH_2)_2$-⌬-SO_3H	always charged
Benzenesulfonic Acid High Load	$Si(CH_2)_2$-⌬-SO_3H	always charged
*** SCX (DVB / Styrene)		

Copolymeric (Mixed Phase)**

SORBENT	STRUCTURE
Aminopropyl + C8	$Si(CH_2)_3NH_3$+ $Si(CH_2)_7CH_3$
Quaternary Amine + C8	$Si(CH_2)_3N^+(CH_3)_3$+ $Si(CH_2)_7CH_3$
Carboxylic Acid + C8	$SiCH_2COOH$+ $Si(CH_2)_7CH_3$
Propylsulfonic Acid + C8	$Si(CH_2)_3SO_3H$+ $Si(CH_2)_7CH_3$
Benzenesulfonic Acid + C8	$Si(CH_2)_2$-⌬-SO_3 H+ $Si(CH_2)_7CH_3$
Cyanopropyl + C8	$Si(CH_2)_3CN$+ $Si(CH_2)_7CH_3$
Cyclohexyl + C8	Si-⬡ + $Si(CH_2)_7CH_3$

** UCT manufactures true copoymeric sorbents by dually reacting their high purity silicas. The product is not a mixed bed sorbent.
***Hydrated DVB / Styrene cross linked sorbent

96 Well Plates

Sorbent	Part Numbers	Amount Sorbent per Well, mg
*ENDCAPPED C18	WORCEC18105	50
	WORCEC1811	100
	WORCEC1812	200
	WORCEC1813	300
*ENDCAPPED C8	WORCEC08105	50
	WORCEC0811	100
	WORCEC0812	200
	WORCEC0813	300
*ENDCAPPED C4	WORCEC04105	50
	WORCEC0411	100
	WORCEC0412	200
	WORCEC0413	300
*ENDCAPPED C2	WORCEC02105	50
	WORCEC0211	100
	WORCEC0212	200
	WORCEC0213	300
CYCLOHEXYL	WORCYH105	50
	WORCYH11	100
	WORCYH12	200
	WORCYH13	300
PHENYL	WORPHY105	50
	WORPHY11	100
	WORPHY12	200
	WORPHY13	300
SILICA	WORSIL105	50
	WORSIL11	100
	WORSIL12	200
	WORSIL13	300
DIOL	WORDIOL105	50
	WORDIOL11	100
	WORDIOL12	200
	WORDIOL13	300
CYANOPROPYL	WORCYN105	50
	WORCYN11	100
	WORCYN12	200
	WORCYN13	300
Florisil PR, 60-100 MESH	WORFLSPR05	50
	WORFLSPR1	100
	WORFLSPR2	200
	WORFLSPR3	300
Florisil 100-200 MESH, GRADE A		
	WORFLSA05	50
	WORFLSA1	100
	WORFLSA2	200
	WORFLSA3	300
ALUMINA-ACID	WORALA05	50
	WORALA1	100
	WORALA2	200
	WORALA3	300

* Also available: C3; C5; C6; C7; C10; C12; C20; C30 All hydrophobic phases are offered in endcapped and unendcapped phases

96 Well Plates

Sorbent	Part Numbers	Amount Sorbent per Well, mg
ALUMINA-NEUTRAL	WORALN05	50
	WORALN1	100
	WORALN2	200
	WORALN3	300
ALUMINA-BASE	WORALB05	50
	WORALB1	100
	WORALB2	200
	WORALB3	300
AMINOPROPYL	WORNAX105	50
	WORNAX11	100
	WORNAX12	200
	WORNAX13	300
N-2 AMINOETHYL	WORPSA105	50
	WORPSA11	100
	WORPSA12	200
	WORPSA13	300
DIETHYLAMINO	WORDAX105	50
	WORDAX11	100
	WORDAX12	200
	WORDAX13	300
**QUATERNARY AMINE	WORQAX105	50
	WORQAX11	100
	WORQAX12	200
	WORQAX13	300
CARBOXYLIC ACID	WORCCX105	50
	WORCCX11	100
	WORCCX12	200
	WORCCX13	300
PROPYLSULFONIC ACID	WORPCX105	50
	WORPCX11	100
	WORPCX12	200
	WORPCX13	300
BENZENESULFONIC ACID	WORBCX105	50
	WORBCX11	100
	WORBCX12	200
	WORBCX13	300
BENZENESULFONIC ACID HIGH LOAD	WORBCXHL105	50
	WORBCXHL11	100
	WORBCXHL12	200
	WORBCXHL13	300
AMINOPROPYL + C8	WORNAX205	50
	WORNAX21	100
	WORNAX22	200
	WORNAX23	300
QUATERNARY AMINE + C8	WORQAX205	50
	WORQAX21	100
	WORQAX22	200
	WORQAX23	300
CARBOXYLIC ACID + C8	WORCCX205	50
	WORCCX21	100
	WORQAX22	200
	WORQAX23	300

**Available with chloride, acetate or hydroxide counter ion.

96 Well Plates

Sorbent	Part Numbers	Amount Sorbent per Well, mg
PROPYLSULFONIC ACID + C8	WORPCX205	50
	WORPCX21	100
	WORPCX22	200
	WORPCX23	300
BENZENESULFONIC ACID + C8		
	WORBCX205	50
	WORBCX21	100
	WORBCX22	200
	WORBCX23	300
CYANOPROPYL + C8	WORCNP205	50
	WORCNP21	100
	WORCNP22	200
	WORCNP23	300
CYCLOHEXYL + C8	WORCYH205	50
	WORCYH21	100
	WORCYH22	200
	WORCYH23	300
DIOL + C18	WORDIOL305	50
	WORDIOL31	100
	WORDIOL32	200
	WORDIOL33	300
***SAX (DVB/STYRENE CROSSLINKED)		
	WORPSAX05	50
	WORPSAX1	100
	WORPSAX2	200
	WORPSAX3	300
***SCX (DVB/STYRENE CROSSLINKED)		
	WORPSCX05	50
	WORPSCX1	100
	WORPSCX2	200
	WORPSCX3	300

METHOD DEVELOPMENT PLATES	Amount Sorbent per Well, mg
HYDROPHOBIC	*20 TO 600*
HYDROPHILIC	20 TO 600
ANION EXCHANGE	20 TO 600
CATION EXCHANGE	20 TO 600
COPOLYMERIC	20 TO 600
CUSTOM	20 TO 600

***Hydrated DVB/STYRENE are all silica phases are standard particle size 40-60µm, 60Å pore size, limited stock of smaller and larger silica phases, please call for availability

Matrix - Assisted Laser Desorption / Ionization Mass Spectrometry of Amine Functionalized Polystyrenes

Frederick J. Cox, Arnab Dasgupta, Murray V. Johnston

University of Delaware, Newark, Delaware

Overview

Purpose

- *Characterize amine functionalized polystyrenes of varying molecular weights*
- Determine effects of modifying functionality between tertiary and quaternary amine on experimental polymer *distributions*

Methods

- Matrix assisted laser desorption / ionization is used to obtain polymer distributions and molecular weight information
- Molecular weight distributions are compared for samples of differing amine functionality, and using different ionization modes

Results

- *Amine functionalized polystyrenes ionize by multiple pathways*
- *Similar average molecular weights are observed for both* quaternary and tertiary amine
- Ionization efficiency is greatly increased *by quaternization*

Introduction

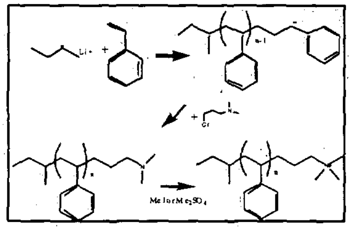
Ionization of most non-polar polymers in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry requires, in addition to a suitable matrix, a metal cation by which a charge can be attached to an oligomer. However, by attaching a suitable charged functional group, oligomers not normally amenable to analysis can be ionized without use of a metal cation[1]. Furthermore, end groups have been observed to significantly change MALDI response depending on their functionality[2]. Because amine functionalized polystyrenes can be ionized in a traditional cationized form, protonated / deprotonated, and a quaternary "covalently" charged form, they provide a useful model system for comparing the effects of such functional groups on MALDI ionization. Here, we compare the spectra of the tertiary and quaternary amine polystyrenes, with and without cationization, and examine the apparent differences in ionization efficiency and average molecular weights arising from functionalization.

Experimental

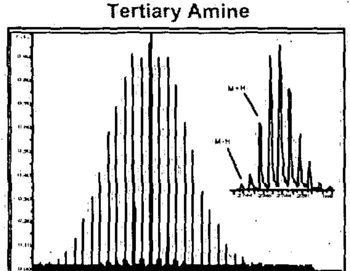
- Mass spectra were acquired on a Bruker Biflex III MALDI time-of-flight mass spectrometer in reflectron mode using delayed extraction. Pulsed ion deflection was used to avoid matrix signal saturation
- Dithranol matrix and AgTFA were obtained from Aldrich. All solutions were prepared in toluene at 4 mg/mL for matrix, salt, and analyte. Solutions were premixed at a 8:1:4 volume ratio and applied to the probe, unless otherwise noted
- Data analysis was performed with Bruker XMASS software; peaks were integrated using the "Integ" module
- EXCEL and MATLAB were used for calculating average molecular weights and deconvolution of overlapping isotopic distributions

Functionalized Polystyrenes

- Amine end functionalized polystyrenes (PS)
- M_n = 1500 to 40 000
- *Anionic polymerization of styrene followed* by addition of propyl amine chloride in presence of LiCl in inert *atmosphere*[3]
- Quaternization by reaction with methyl iodide or dimethyl sulfate

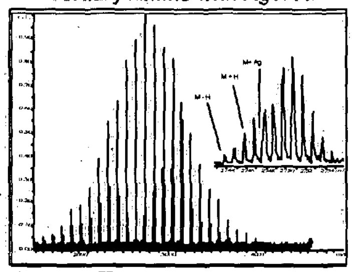


Tertiary Amine

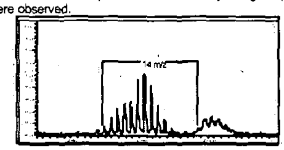


The spectrum of a M_n = 2600 tertiary amine sample with *dithranol matrix* is shown above. Because of the overlap of the M-H and M+H isotope distributions, and the possibility of an M· ion, theoretical distributions of each ion were fit to the experimental integrated areas using least squares analysis. Only M-H and M+H ions were determined to be present in a 1:6 ratio. The M+H ion likely forms by protonation of the amine, while the M-H ion is likely a product of the loss of H_2 from the M+H ion caused by the formation of an imine. Well-resolved series of ions of weak intensity were observed at M + 17 m/z, possibly an ammonia adduct, and M - 13 m/z (or M + 91m/z), which has not been determined.

Tertiary Amine with AgTFA



Addition of AgTFA to the previous tertiary amine sample results in the formation of the M_{-1}+Ag ion in addition to the previously observed M-H and M+H ions. The average ratio of the M-H to M+H to M_{-1}+Ag ion was determined to be 1:2:7 by a least squares fit of the theoretical isotopic distribution to the experimental. No doubly charged species were observed.



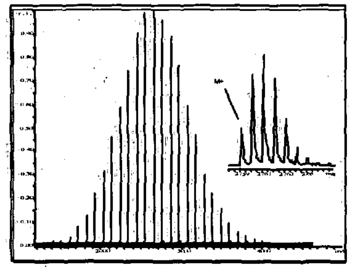
A partially resolved secondary ion series was observed 14 m/z higher than M (or 90 m/z below) only in tertiary amine spectra containing AgTFA. However, the isotopic distribution does not support inclusion of Ag in the ion. Intensity increased with higher laser power, and M_i values were equivalent to that of the major series. Because the ionization potentials of the silver atom and the tertiary amine are comparable, it is possible that charge exchange occurs, leading to an M+ ion with further reaction to the observed ion. The identity of the ions is yet to be determined.

Average Molecular Weights

Estimated weight	MALDI Tertiary	MALDI w/silver	MALDI Quaternary
1800	1689	1764	1662
2600	2590	2711	2651
11000	14151	14347	14302

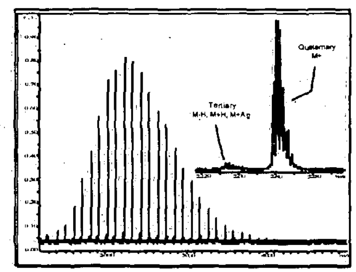
Integrated peak areas were obtained from a minimum of *three spectra each (one each for 11000 MW sample)* of the tertiary amine, tertiary amine with AgTFA, and the quaternary *amine, and used to calculate number* average molecular weight (M_n) of the polymer distributions. The increase in mass due to silver cationization was not subtracted out prior to calculation. Because GPC can be performed only on the *tertiary amines samples*, molecular weights are estimated. Values obtained by all three ionization methods compare *well among each sample.* Both differing functionalization and ionization mode appear to have little effect on average molecular weight.

Quaternary Amine



Spectra of the quaternary amine give only the single M+ peak *for each oligomer when analyzed with matrix. Addition* of AgTFA resulted in no visible change in spectra other than silver clustering. *Signal was observed at both lower laser* powers than the tertiary amine, and with significantly greater *intensity at equivalent power. No appreciable* secondary ion series were observed. Without matrix, M+ ions can be *observed, but with substantial decrease in signal and* only at highly disperse "sweet spots".

Ionization Efficiency



Equimolar quantities of both quaternary and tertiary amines were blended (M_n = 2600, above) with dithranol and AgTFA in order to obtain information on relative ionization efficiency. Integrated areas of each oligomer for the tertiary and quaternary components were summed and averaged using a minimum of three spectra. The ratio of the quaternary to tertiary components was found to be 20:1. Ionization efficiency of the tertiary amine is thus 5% of the quaternary, posing significant problems for monitoring conversion of tertiary to quaternary amine.

Conclusions

- Amine functionalized polystyrenes form M-H, M+H, and *M_{-1}+Ag ions upon analysis by MALDI*
- Amine PS quaternized with a methyl group forms only the M+ ion, regardless of the addition of common cationizing agents, and does not *require* matrix for ionization
- Experimentally determined average molecular weights are similar between tertiary and quaternized amine functionalized PS, with and without cationizing agents
- Ionization efficiency of the quaternary amine is roughly 20 times that of the tertiary amine
- *Functionalization increases* ease of MALDI-MS analysis by increasing ionization efficiency, decreasing spectral complication, and eliminating the need for a cationizing agent

Future Work

Additional samples of the type described here have been synthesized and await further investigation. A diamine end functionalized PS and a dual end functionalized PS are currently being synthesized in order to investigate the effect of multiple functional group attachment to the PS, and the effect of a dual charge, if possible, on the mass spectra. Further examination of lower intensity ion series and metastable ions is to be performed, including the use of post - source decay for identification.

Acknowledgements

- Robert Fuedale *for helpful* assistance with MATLAB
- Burnaby Munson is thanked for his hospitality and fruitful discussion

Funding

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- A. Dasgupta was supported by funding from the ACS Petroleum Research Fund, NSF grant 9973740, and the U.S. Army through the Center of Composites Materials at the University of Delaware

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PH - 014

EXTRACTION OF VITAMIN B_{12} FROM MULTIVITAMIN TABLETS

FIELD
Pharmaceutical/Clinical

SAMPLE
Vitamin B_{12} (Cobalamin)

MATRIX
Multivitamin Tablets

EXTRACTION COLUMN
BAKERBOND spe™ Quaternary Amine (N^+), 3 mL (500 mg); Phenyl (C_6H_5), 3 mL (500 mg); Filtration Column, 3 mL

SAFETY AND PROTECTIVE EQUIPMENT
Goggles and face shield, lab coat and apron, vent hood, proper gloves, Type B fire extinguisher.

SAMPLE PREPARATION
Add 10 mL extracting solution (1.3 g dibasic sodium phosphate, 1.2 g citric acid monohydrate, 1 g sodium metabisulfite/100 mL of HPLC grade water) to 1 tablet weight of multivitamin tablet powder in a 25 mL low actinic flask. Sonicate for 2 minutes. Shake on a mechanical shaker for an additional 15 minutes. Allow flask to stand undisturbed for 2 minutes before sampling.

STANDARD PREPARATION
Dissolve appropriate amount of B_{12} standard in a given volume of extracting solution. Make proper dilutions with extracting solution to give the B_{12} concentration expected in the sample solution. Treat standard as if it were a sample for the remainder of the procedure.

COLUMN CONDITIONING
Condition both the quaternary amine and phenyl columns with 3 mL methanol, followed by 3 mL HPLC grade water, followed by 3 mL extracting solution. With vacuum off, fill each column with extracting solution. Place adapter on top of each column and fit the quaternary amine column into the adapter on top of the phenyl column. Attach a 6 mL filtration column to the adapter on top of the quaternary amine column.

SAMPLE ADDITION/WASH
Transfer 2 mL of sample solution to the filtration column and aspirate solution through entire column assembly. Wash columns with 2 x 1 mL of extracting solution and remove the filtration and quaternary amine columns. Wash the phenyl column with 1 mL HPLC grade water. Dry interior of column with a cotton swab (be sure to remove all water droplets) and air dry

column under vacuum for 5 minutes. Wash the column with 3 mL hexane, followed by 1 mL methylene chloride, followed by 2 ml acetonitrile (dried over anhydrous sodium sulfate), followed by 2 mL acetonitrile/ methanol (dried over anhydrous sodium sulfate) (95:5). Do not allow columns to dry between solvent additions. Air dry column under vacuum for 1 minute after the acetonitrile/ methanol wash.

SAMPLE ELUTION

Elute with 2 x 0.5 mL methanol/HPLC grade water (9:1) collecting the eluate in a 1 mL volumetric flask. Dilute to volume with the eluting solvent and mix well.

PRODUCT LIST

Product Number	Description
7091-03	BAKERBOND spe™ Quaternary Amine (N^+), 3 mL (500 mg)
7095-03	BAKERBOND spe™ Phenyl (C_6H_5), 3 mL (500 mg)
7121-06	Filtration Column, 6 mL
9017-03	Acetonitrile, 'BAKER ANALYZED'® HPLC
7122-00	Adapter
0110-01	Citric Acid, Monohydrate, 'BAKER ANALYZED'® ACS Reagent
9304-03	Hexanes, 'BAKER ANALYZED'® HPLC
9093-03	Methanol, 'BAKER ANALYZED'® HPLC
9315-03	Methylene Chloride, 'BAKER ANALYZED'® HPLC
3828-01	Sodium Phosphate, Dibasic, 'BAKER ANALYZED'® ACS Reagent
3891-01	Sodium Sulfate, Anhydrous, 'BAKER ANALYZED'® ACS Reagent
3552-01	Sodium Metabisulfite, 'BAKER ANALYZED'® ACS Reagent
4218-03	Water, 'BAKER ANALYZED'® HPLC

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Product Catalog & Information

Research & Analysis

Catalog : Distributors : Product Literature : Technical Library
Data updated 5/17/2003

BAKERBOND spe™ Quaternary Amine (N^+) Disposable Extract Columns

Product Number	Group Code	Units per Case	Pkg Size	Container Type	Unit Price	Case Price	Sorbent Weight	Column Size	C
7091-01	SPE	---	1 BX	---	$ 174.10	N/A	100 mg	1 mL	
7091-03	SPE	---	1 BX	---	$ 151.20	N/A	500 mg	3 mL	
7091-07	SPE	---	1 BX	---	$ 135.80	N/A	---	---	
7091-27	SPE	---	1 PK	Lined Fiber Dr	$ 1374.00	N/A	---	---	
7091-29	SPE	---	1 PK	Lined Fiber Dr	$ 1285.30	N/A	---	---	

CAS Number: **126850-06-4**
Storage Color Code: **Orange**

Material Safety Data Sheet

	Health	Flammability	Reactivity
SAF-T-DATA Rating	2	0	0

Potassium channel receptor site for the inactivation gate and quaternary amine inhibitors

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Howard Hughes Medical Institute, Laboratory of Molecular Neurobiology and Biophysics, Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

Many voltage-dependent K$^+$ channels open when the membrane is depolarized and then rapidly close by a process called inactivation. Neurons use inactivating K$^+$ channels to modulate their firing frequency. In Shaker-type K$^+$ channels, the inactivation gate, which is responsible for the closing of the channel, is formed by the channel's cytoplasmic amino terminus. Here we show that the central cavity and inner pore of the K$^+$ channel form the receptor site for both the inactivation gate and small-molecule inhibitors. We propose that inactivation occurs by a sequential reaction in which the gate binds initially to the cytoplasmic channel surface and then enters the pore as an extended peptide. This mechanism accounts for the functional properties of K$^+$ channel inactivation and indicates that the cavity may be the site of action for certain drugs that alter cation channel function.

The presence of an inactivation gate causes a K$^+$ channel to close spontaneously after opening induced by membrane depolarization (Fig. 1a). The inactivation gate in Shaker-type K$^+$ channels is formed by the first 20 amino acids on the N terminus of the α-subunit[1,2] or β-subunit[3], located on the intracellular side of the membrane (Fig. 1c, d). The essential chemical characteristics that enable the N terminus to act as a gate are that the first approximately 10 amino acids tend to be hydrophobic (hydrophobic region) and the remaining 10 hydrophilic with excess positive charge (hydrophilic region)[4,5] (Fig. 1d).

Four lines of evidence support the idea that the inactivation gate binds to the pore. First, inactivation occurs only after the voltage-dependent gate opens, as if the opening of the pore exposes a receptor for the gate[6]. Second, inactivation is produced by the binding of only one gate, presumably to the single pore opening, even though K$^+$ channels have four gates (N termini), by virtue of their homotetrameric architecture[7,8]. Third, high concentrations of extracellular K$^+$ reduce inactivation, as if K$^+$ ions traversing the pore push the gate from its intracellular site[9]. Fourth, inactivation mimics the action of quaternary amines, which are thought to be pore blockers[10,11] (Fig. 1a, b). Furthermore, quaternary amines compete with the gate to inhibit K$^+$ current[11].

How does the N-terminal gate interact with the pore to cause inactivation? Studies using mutagenesis have highlighted amino acids that might be expected to reside near the intracellular pore opening, for example, those between the fourth and fifth membrane-spanning segments, which connect the voltage sensor to the pore module[12]. In addition, the structures of inactivation gates have been analysed by NMR spectroscopy[13,14]. Together, these approaches have led to a picture of an inactivation 'domain' capping the pore's intracellular face[13,15]. This picture is reasonable, but the quantitative details of earlier work are more compatible with a different structural view[4,5].

Pore occlusion by an extended N terminus

Structural studies have shown that the pore of a voltage-dependent K$^+$ channel opens to the cytoplasm between the T1 domain and the transmembrane channel[16–18] (Fig. 1c). On the basis of mutant cycle analysis, the inactivation gate was proposed to reach its site of action by entering the openings above the T1 domain[16] (Fig. 1c). Here we ask, where is the inactivation gate's site of action? To address this question, we studied inactivation mediated by the β1 subunit inactivation gate attached to the β2 core (β12)[16,19] (Fig. 1c, d). The β12 subunit was expressed in *Xenopus* oocytes with the $K_v1.4$ channel α-subunit, a mammalian homologue of the Shaker K$^+$ channel. The $K_v1.4$ α-subunit contained a deletion in its own N terminus (1.4-IR; see Methods) to ensure that inactivation would be mediated only by the β12 inactivation gate[16,19] (Fig. 1a). The inactivation process was parameterized by the inactivation time constants τ_{on}, τ_{off} and the ratio τ_{on}/τ_{off}, referred to as K_d (the dissociation constant: see Fig. 2a and Methods).

Mutations to alanine or to valine (position 6) in the inactivation gate affected K_d, a measure of the apparent affinity of the gate for its receptor, in a manner very consistent with previous findings on the Shaker K$^+$ channel[1,2,4,5] (Fig. 2a). Mutations in the hydrophobic region had large energetic effects, expressed as changes in the apparent dissociation rate constant ($1/\tau_{off}$), whereas those in the hydrophilic region had more modest effects and altered both the apparent association ($1/\tau_{on}$) and dissociation rate constants. The importance of residues very close to the N terminus, in the hydrophobic region, is emphasized by the observation that a peptide corresponding to the first four amino acids alone (with a carboxy-terminal amide rather than a carboxylic acid) retains some ability to inhibit K$^+$ current (Fig. 2b).

The sixth membrane-spanning segment of voltage-dependent K$^+$ channels corresponds to the inner helix of the KcsA K$^+$ channel, which lines the pore on the intracellular side of the selectivity filter[20]. This region of the pore forms a 10 Å-wide cavity at the membrane centre, the central cavity, that gradually tapers to about 4 Å diameter near the cytoplasmic opening. The pore-lining surface is predominantly hydrophobic in this region of the channel. We mutated amino acids that were predicted to point towards the pore on the basis of the KcsA K$^+$ channel structure. Five mutations out of six (to alanine) had significant effects on the inactivation gate K_d (Fig. 2c). The effects at positions 551 and 554, corresponding to cavity-lining residues 100 and 103 in KcsA, were so large that the K_d shown is an approximation based on the residual current measured after inactivation (see Methods). We used double-mutant cycle analysis of inactivation gate and inner helix mutations to assess the proximity of amino acids in the inactivated state (Fig. 2d). The Val 3 mutation

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on the gate was coupled to the Val 558 and Val 562 mutations on the inner helix, and the Ile 5 mutation was coupled to the Tyr 569 mutation. It was not possible to determine accurately the mutant cycle coupling energies involving positions 551 and 554, but the results at other positions imply that the inactivation gate lies in an extended conformation in the inner pore (Fig. 2d, e).

The central cavity binds hydrophobic cations

The above results support the simple conclusion that the inactivation gate apparently enters the inner pore and lodges its N terminus into the central cavity. To further support this hypothesis, we next made use of the fact that quaternary ammonium inhibitors mimic the action of the inactivation gate[10,11] (Fig. 1a, b). The KcsA K^+ channel was crystallized in the presence of tetrabutylammonium (TBA) and an electron-dense analogue, tetrabutylantimony (TBSb). TBSb is chemically very similar to TBA and blocks K^+ channels accordingly (Fig. 3a). The heavy atom Sb provides the distinct advantage of easy identification in an electron density map. Data were collected from each crystal and a difference electron density map $(F_{TBSb}-F_{TBA})$PHIcalc was calculated (Fig. 3b; see Methods). The strong electron density peak reveals the binding site for TBA in the cavity. Refinement of the channel–TBA complex indicates that the presence of TBA in the cavity has little influence on the structure. Compared to the structure without TBA, the inner helices are drawn inwards towards the centre by a few tenths of an angstrom at the level of the cavity and are unchanged below the cavity (Protein Data Bank code 1J95).

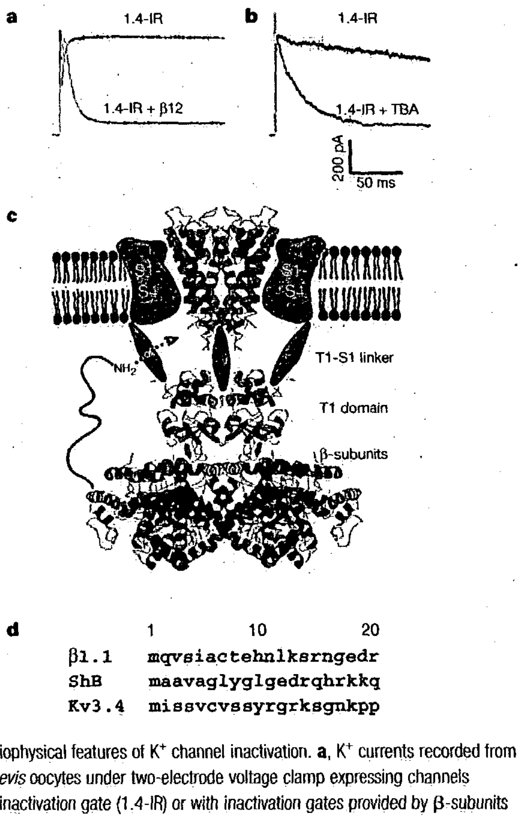

Figure 1 Biophysical features of K^+ channel inactivation. **a**, K^+ currents recorded from *Xenopus laevis* oocytes under two-electrode voltage clamp expressing channels without an inactivation gate (1.4-IR) or with inactivation gates provided by β-subunits (1.4-IR + β12). The maximum current value is 1.4 μA and 2 μA for noninactivating and inactivating currents, respectively. Time scale is given in **b**. **b**, K^+ currents from 1.4-IR channels recorded from an excised, inside-out patch under voltage clamp in the absence (1.4-IR) or presence of 10 μM TBA (+ TBA). **c**, Composite model of a voltage-dependent K^+ channel[16]. The α-subunit is shown in blue and the β-subunit in red. The pore is represented by the KcsA K^+ channel[20] and the T1-β complex is from ref. 16. The structures of the voltage sensor (S1–S4) and linker (T1–S1) connecting the voltage sensors to the T1 domain are unknown. An N-terminal inactivation gate is shown entering a lateral opening to gain access to the pore. The image was prepared by Molscript[30] and raster-3D[31]. **d**, Sequence alignment shows inactivation gates from $K_v\beta1.1$ (accession number CAA 50000), Shaker B (accession number CAA 29917) and $K_v3.4$ (accession number XP_002146).

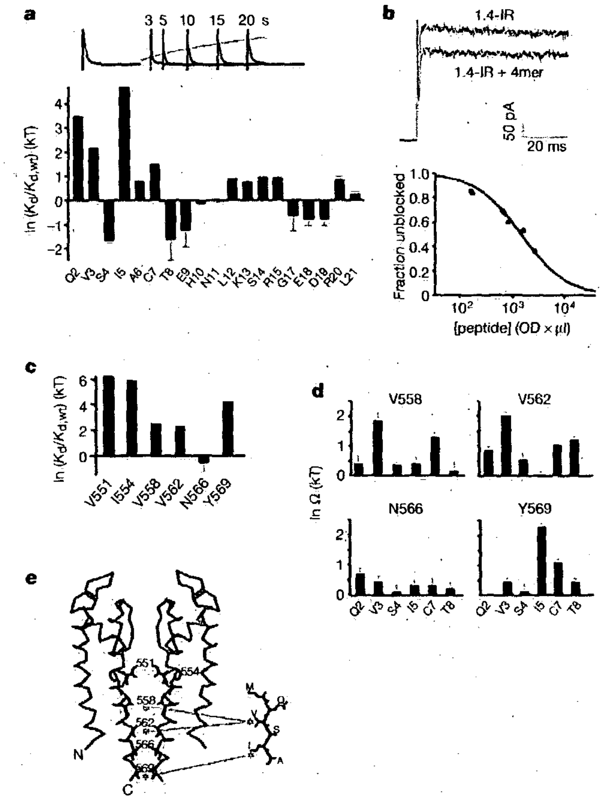

Figure 2 Mutational analysis of the inactivation gate–receptor interaction. **a**, Top, inactivation rates in $K_v1.4$-IR + β12 channels determined by analysis of currents during a depolarizing pulse from −80 mV to +60 mV and recovery of current during a paired-pulse protocol[7]. τ_{on} (5.0 ± 0.3 ms) is the short time constant of a double exponential fit to current inactivation (red line) and τ_{off} (11 ± 0.7 s) is the time constant describing recovery in paired pulses (black line). Bottom, alanine-scanning mutagenesis of the inactivation gate. K_d, defined as τ_{on}/τ_{off}, was determined for $K_v1.4$-IR + β12 channels with mutations to alanine or valine (position 6) at positions 2–21 in the β12 inactivation gate. The K_d values, normalized by that for wild type, are shown. Error bars represent s.e.m. from ≥5 oocytes. **b**, Top, current recorded from an excised, inside-out patch containing 1.4-IR channels without (1.4-IR) and with (+ 4mer) a peptide corresponding to the first four amino acids of the β12 inactivation gate. Bottom, dose–response curve showing current inhibition by the 4mer peptide as a function of concentration in units of optical density × volume. Data were collected from 12 patches. **c**, Alanine-scanning mutagenesis of pore-lining residues. The K_d for six pore-lining mutations to alanine, normalized by the wild-type K_d, is shown. Error bars represent s.e.m. from 3–7 oocytes. **d**, Double-mutant cycle analysis between pore-lining residues and residues on the inactivation gate. Ω calculated for six residues on the inactivation gate and four residues on the pore-lining helix is shown. Inactivation did not occur when Y569A on $K_v1.4$-IR was paired with Q2A on β12. The approximate K_d determination for V551A and I554A mutations did not allow determination of Ω. Error bars show the s.e.m. measured in ≥5 oocytes. **e**, Summary of mutational analysis. Left, two diagonally positioned KcsA K^+ channel subunits are shown in C_α trace, with pore-lining residues of the KcsA K^+ channel shown as sticks but labelled according to $K_v1.4$ residue numbering. Right, an extended strand model for the first six residues of the inactivation gate with side chains shown as sticks. Green and purple connecting lines identify coupled residues in the mutant cycle analysis.

-32-

Inner helix mutations in the 1.4-IR channel alter inactivation and inhibition by TBA in a roughly similar manner (Fig. 3c). This finding further supports a common mechanism in which the inactivation peptide, like TBA, enters the pore and reaches the cavity. One notable difference between the inactivation gate and TBA is evident near the bottom of the inner helix, where the Y569A mutation has a relatively larger effect on inactivation. This difference is reasonable, however, as the inactivation gate comprises 20 amino acids and presumably interacts with the pore all the way from the cavity to the intracellular opening. A comparison of the size of TBA and the inactivation peptide leads us to propose that probably only the first three amino acids of the inactivation peptide bind in the cavity (Fig. 3d). These amino acids are generally hydrophobic in the inactivation gates (the β1 inactivation gate is unusual in having a glutamine in the second position) and contain the N-terminal amino group, and are therefore chemically similar to TBA.

Our data lead us to propose that inactivation occurs through the interaction of the K^+ channel with a fully extended N-terminal peptide. The hydrophobic region of the peptide would extend from the cavity to the intracellular entryway, while the hydrophilic peptide region would emerge from the pore and interact with the aqueous protein surfaces lining the cage formed above the T1 domain (Fig. 1c). This configuration makes good chemical sense as the cavity and inner pore are lined by hydrophobic amino acids and the T1–S1 linkers outside the pore contain many acidic amino acids that would interact favourably with the inactivation peptide's multiple basic residues. This picture is consistent with the deduction of Aldrich *et al.* that the hydrophobic region must be 'buried in a hydrophobic environment' and that the hydrophilic region is important for 'long-range electrostatic interactions'[4,5].

Sequential steps of inactivation

The idea that the N-terminal gate snakes into the inner pore as an extended peptide contradicts proposals of a structured inactivation domain docking superficially on the intracellular opening[13,15]. The complex pathway to the intracellular pore opening indicates that the peptide probably reaches its final inactivating configuration through sequential binding steps (Figs 1c, 4a). For example, it is reasonable to imagine that the peptide first binds on the protein surface outside the pore, producing a preinactivated state, and then inserts its hydrophobic region to block the pore as outlined in the kinetic scheme (Fig. 4a):

$$O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I$$

The channel would conduct ions in the open (O) and preinactivated (O') states and become blocked in the inactivated (I) state. The effects of mutations on the forward (onset of inactivation) and backward (recovery from inactivation) rates can offer insight into the relative rates in such a sequential reaction scheme. Here and in previous studies, mutations in the hydrophobic region of the peptide affected mainly the apparent backward rate, I → O, whereas mutations in the hydrophilic region affected modestly both the forward and backward rates[4,5] (Fig. 4b). These observations make perfect sense if k_1 is small and $k_2 \gg k_{-1}$, conditions under which the forward rate O → I will be dominated by k_1 and the backward rate I → O will be related to $k_{-1} \times (k_{-2}/(k_2 + k_{-2}))$. Hydrophobic region mutations, by altering k_2 and k_{-2}, will affect only the backward rate, and hydrophilic region mutations, by altering k_1 and k_{-1}, will affect both rates. We therefore suggest that the first transition to form the

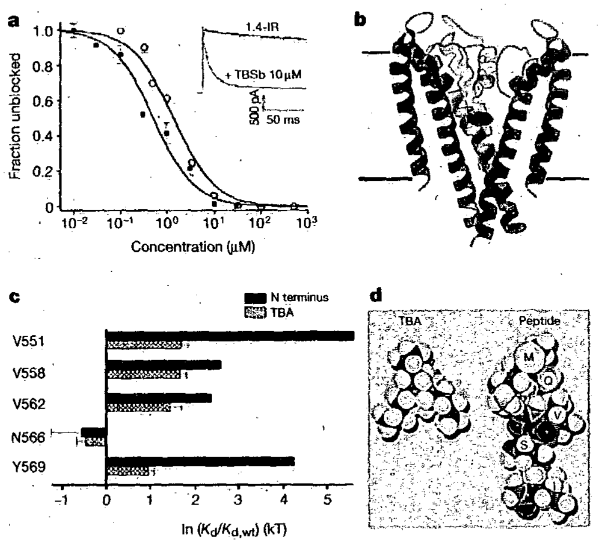

Figure 3 TBA binds in the cavity of the KcsA K^+ channel. **a**, Functional study of TBA and TBSb: currents from excised, inside-out patches were recorded under different TBA (open circles) or TBSb (filled squares) concentrations and fraction of residual current is plotted against inhibitor concentration. Smooth curves correspond to the Langmuir equation with K_d for TBA and TBSb of 1.5 μM and 0.7 μM, respectively. Inset, example currents with and without TBSb (10 μM). **b**, Ribbon diagram of three subunits of the KcsA K^+ channel. The red surface contoured at 6σ is the largest positive peak (maximum 18σ) present in a difference electron density map calculated at 4.0 Å with coefficients F_{TBSB}–F_{TBA} and model phases from a refined model with TBA omitted. **c**, Effects of inner helix (S6) mutations on TBA block and inactivation. For pore-lining mutations on the inner helix, the natural logarithm of K_d for TBA and the inactivation gate (normalized to that of wild type) are plotted. Error bars for inactivation are s.e.m. from 3–7 oocytes. Error bars for TBA block are s.e.m. from ≥5 patches. **d**, CPK models of TBA (left) and the first five residues of the N-terminal inactivation gate (right). The inactivation gate is not at its most extended conformation, but the volume of the first three amino acids is comparable to that of TBA.

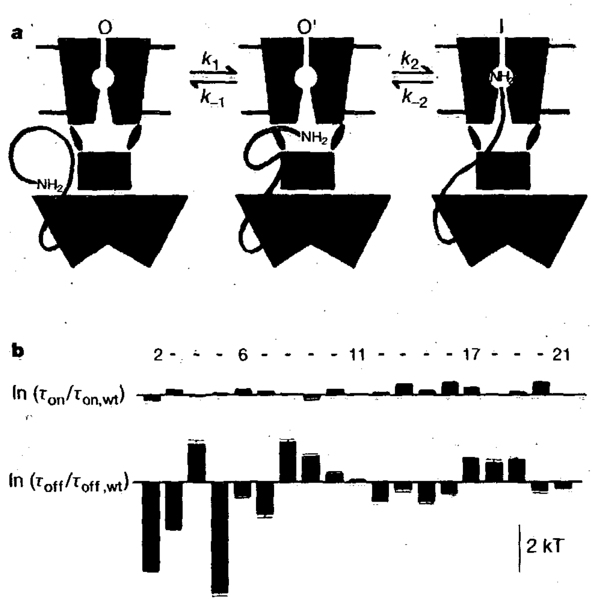

Figure 4 A structural model for the mechanism of inactivation. **a**, Open K^+ channel with three different configurations of the N-terminal inactivation gate shown attached to the β-subunit. For clarity, one inactivation gate is shown instead of four. O, open channel with its N terminus before docking; O', open channel with its N terminus bound to the hydrophilic protein surface; I, open channel with its N terminus entering the cavity (blocking the channel). **b**, The effect of N-terminal mutations on time constants of inactivation (τ_{on} and τ_{off}). The natural logarithms of inactivation time constants (normalized to that of the wild type) are plotted. Numbers above the bars represent inactivation gate residue numbers (Fig. 2). Error bars are s.e.m. from ≥5 oocytes.

preinactivated state can be rate limiting and that the final plugging transition can be fast, at least in some K^+ channels.

One prediction made by these rate conditions is that the forward rate of inactivation should be nearly voltage independent, as the rate-limiting step occurs outside the membrane electric field. Voltage-independent inactivation is observed in some K^+ channels[6]. A second prediction of these rate conditions is that if a fraction of channels exists in the preinactivated state before opening of the voltage-dependent gate, they should very rapidly inactivate upon opening. If sufficiently rapid, the inactivation peptide would appear to produce closed-state inactivation. Apparent closed-state inactivation has been documented with different inactivation gates of the Shaker splice variants[4]. Moreover, systematic truncation of the Shaker D peptide hydrophilic region modulated the extent of the apparent closed-state inactivation, as the model in Fig. 4a would predict[4].

Discussion

Our findings lead us to conclude that the hydrophobic central cavity and inner pore of K^+ channels form the receptor site for both the inactivation gate and quaternary ammonium compounds. This conclusion explains the following functional properties of inactivation: a single gate is sufficient to cause inactivation[7,8]; quaternary ammonium compounds compete with the inactivation gate[11]; external K^+ pushes quaternary ammonium ions and the gate out of the pore[9,21]; inactivation is voltage independent[6]; and some K^+ channels appear to exhibit closed-state inactivation[4]. A central cavity receptor for molecules as large as TBA and the inactivation gate also has implications for activation gating conformations in K^+ channels. In the KcsA crystal structure, the pore entryway near the cytoplasm has a diameter of about 4 Å. The diameter is unchanged when TBA is present in the cavity, but the pore must open sufficiently wide for TBA or the inactivation gate to reach the cavity.

Many pharmacological agents that influence cation channel function are both hydrophobic and cationic. On the basis of this study, we suggest that many of these agents bind in the cavity[22,23]. □

Methods

Mutagenesis and expression

We used rat $K_v1.4$-IR (residues 111–655, accession number CAA 34133) and rat β12 chimaera (rat β2 core (residues 36–367, accession number CAA 54142) spliced at the N terminus with rat β1 (residues 1–70, accession number CAA 50000))[16]. We introduced point mutations by the QuickChange method (Stratagene) and confirmed them by sequencing the entire complementary DNA insert. We prepared RNA by T7 polymerase transcription and injected it into *Xenopus laevis* oocytes[24].

Electrophysiology

We used a two-electrode voltage clamp (OC-725B, Warner Instrument Corp.) to record K^+ currents from oocytes 1–2 days after injection with messenger RNA. Electrodes had a resistance of ~0.5 MΩ (3 M KCl). The bath solution contained (in mM): NaCl 96, KCl 2, $CaCl_2$ 0.3, $MgCl_2$ 1 and HEPES 5 at pH 7.4. Oocytes were held at −80 mV and stepped to +60 mV for 200 ms to elicit K^+ current. Data collection and analysis methods are described in ref. 16.

We recorded patch-clamp currents from inside-out, excised patches from oocytes 3–5 days after injection. Electrodes were drawn from patch glass (PG150T-10, Warner Instrument Corp.) and polished to a resistance of 0.6–1 MΩ. The pipette solution (outside) contained (in mM): KCl 140, $MgCl_2$ 2 and HEPES 10 at pH 7.4. The bath solution (inside) contained (in mM): KCl 140, EGTA 5, $MgCl_2$ 2 and HEPES 10 at pH 7.4. K^+ currents were elicited by holding the patch at −100 mV and stepping to +60 mV for 300 ms. Solution exchange was achieved by gravity flow. Analogue data from an Axopatch-1D amplifier (Axon Instruments) were filtered (3 kHz, −3 dB) by an 8-pole Bessel filter (Frequency Devices), digitized at 20 kHz and stored on a PC hard disk.

Synthetic peptide and blockers

Inactivation peptide (4-mer) was synthesized by the Protein/DNA technology Center of the Rockefeller University. Rink amide resin was used to ensure that the C terminus of the peptide was amidated. We purified peptide by reversed-phase high-performance liquid chromatography, dissolved it in bath solution and added it directly to the bath. The peptide amount used was quantified by optical density (at 215 nm) × vol (μl).

We purchased TBA and TBSb from Kodak and Aldrich, respectively. We dissolved TBA or TBSb in bath solution and perfused it onto an inside-out patch by gravity flow.

Crystallography

KcsA was expressed and purified as described[20]. We incubated the chymotrypsin-cut protein at around 10 mg ml^{-1} in a solution containing 150 mM KCl, 50 mM Tris (pH 7.5), 2 mM DTT and 5 mM *N,N*-dimethyldodecylamine-*N*-oxide with 1 mM of TBSb or 5 mM TBA for 15–30 min at room temperature. Crystals were obtained as described[20]. Data were collected under a stream of boiled-off nitrogen at stations ID-13, ESRF and X-25, National Synchrotron Light Source, Brookhaven National Laboratory. The TBA co-crystal data extends to 2.8 Å with R_{sym} = 7.2%, 93% complete, redundancy ~2 and the TBSb co-crystal data extends to 3.45 Å with R_{sym} = 7.0%, 97% complete, redundancy ~3. The data were processed with Denzo and Scalepack[25]. All other calculations were done with the CCP4 package[26]. The two data sets were scaled together to 4 Å with R_{merge} = 22.5% before the calculation of the difference electron-density map.

Analysis of inactivation, TBA block and double-mutant cycles

We determined the inactivation gate affinity for the channel by taking the ratio τ_{on}/τ_{off}. This definition approximates the equilibrium constant k_{off}/k_{on} with two sources of error. First, even for a two-state process, $\tau_{on}/\tau_{off} = k_{off}/(k_{on} + k_{off})$. Given that in most channels studied $k_{on} >> k_{off}$, this approximation introduces only a small error. Second, the inactivation process is not a two-state process, as discussed. In a separate analysis we modelled inactivation as a three-step reaction with a slow first and rapid second transition and found that our analysis, assuming two states, was sufficient for parameterization of mutational effects. To determine τ_{on}, we fit the inactivating current with a double exponential function and took the fast component (τ < 50 ms, typically > 90% of inactivation) as τ_{on}. To determine τ_{off}, we fit the envelope of recovery in paired pulse experiments to a single exponential function[7]. In some mutant channels, we had to fit recovery with a double exponential function. We took the faster component (τ < 1 s, 50–80% of current) as τ_{off}. Justification for this assignment is based on previous studies showing that the slow component of recovery is due to C-type inactivation[19,27]. We verified this conclusion in two ways, by raising extracellular K^+ concentration to 96 mM, and by introducing a point mutation (K533Y) at the external TEA binding site[9,28]. Both manoeuvres caused the slow component of recovery to disappear, compatible with its designation as the C-type process. In the case of mutations V551A and I554A, τ_{off} was too small to measure accurately and so we estimated the apparent gate affinity from the fraction of current remaining after inactivation (~87% and ~70%, respectively).

To quantify TBA, TBSb or peptide blocking, we plotted the fraction of residual current at the end of a 300-ms pulse against blocker concentration and fitted it with the following equation to obtain K_d, the equilibrium dissociation constant: fraction unblocked = 1/(1 + [blocker]/K_d).

We used the double-mutant cycle parameter Ω, where

$$\Omega = \frac{K_d^{\text{wt,wt}} \times K_d^{\text{mut,mut}}}{K_d^{\text{wt,mut}} \times K_d^{\text{mut,wt}}}$$

to quantify the degree of coupling between two mutants[29]. An Ω value of more than unity indicates that the effects of two mutations are coupled. We used the mean and s.e.m. of K_d to obtain the range of uncertainty on Ω, assuming linear propagation of independent errors through the above equation.

Received 5 March; accepted 19 April 2001.

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Acknowledgements

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Correspondence and requests for materials should be addressed to R.M. (e-mail: mackinn@rockvax.rockefeller.edu). Coordinates have been deposited with the Protein Data Bank under accession code 1J95.

Medline Repository

Quaternary amine --> Tertiary amine

PMID	Sentence	Species
3179614	This would be entropically more favourable for [3H]-mepyramine, a tertiary amine, than for [3H]-QMDP, a quaternary amine.	Guinea Pigs
3024994	However, tertiary amine analogs were substantially less potent than hemicholinium-3 or their quaternary amine analogs.	unknown
1061147	As predicted by this hypothesis, the drugs' effects were seen only after a short time lag, and quaternary amines were less effective than tertiary amines.	Unknown
702324	Only one of seven quaternary amines tested inhibited PAEB uptake at an inhibitor/substrate ratio (I/S) of 7.5, while four out of five tertiary amines significantly decreased Vo at an I/S of 0.75 and all five decreased it at a ratio of 7.5.	unknown
161493	The 3-(CO-NH2), or -(CO-NHOH), substituted pyridinic compounds (nicotinamide, nicotinohydroxamic acid) prevent perfectly dicrotophos-induced beak and legs malformations, in tertiary amine form, but very little in quaternary amine form (methyliodide).	unknown
6808705	The effects of a secondary amine (ketamine), tertiary amines (dibucaine, lidocaine, marcaine, propanidid, diazepam and chlorpromazine) and a quaternary amine (tetraethylammonium bromide, TEA) on mouse 3T3 cell agglutination by concanavalin A (Con A), on patch formation of Con A receptors on the cell surface, and on paracrystal formation by vinblastine in cytoplasm were studied.	unknown
2306637	Rats were injected with either saline; A-4 (40 mg/kg, i.p.), a bis tertiary amine derivative of hemicholinium-3; or A-5 (50 micrograms/kg, i.p.), a bis quaternary amine derivative of hemicholinium-3, 1 h prior to moderate fluid percussion brain injury.	Rats
1421216	Quaternary amine n-propyl-ajmaline induced use-dependent inhibition of CMH-units in lower concentrations than tertiary amine lidocaine.	unknown
8877848	The dissociation constants for two chemically different anticholinergics, the tertiary amine scopolamine and the quaternary amine oxyphenonium, were calculated from inhibition studies of 3H-NMS binding in buffer and plasma.	Cattle Human
9371413	Other electrostatic characteristics found were that the primary amine group of DOPE in cationic liposomes dissociated at high (> 7.9) pHbulk and that a salt bridge was likely between the quaternary amine of DOTAP or DMRIE and the phosphate group of DOPE or DOPC, but not between the tertiary amine of DC-CHOL and the phosphate group of DOPE.	unknown
10479355	Quaternary amine prodrugs resulting from N-phosphonooxymethyl derivatization of the tertiary amine functionality of drugs represents a novel approach for improving their water solubility.	Unknown

-36-

1: Br J Pharmacol. 1988 Jul;94(3):811-22.

Temperature-dependence of the kinetics of the binding of [3H]-(+)-N-methyl-4-methyldiphenhydramine to the histamine H1-receptor: comparison with the kinetics of [3H]-mepyramine.

Treherne JM, Young JM.

Department of Pharmacology, University of Cambridge.

1. The dissociation of [3H]-(+)-N-methyl-4-methyldiphenhydramine ([3H]-QMDP) from the histamine H1-receptor was markedly temperature-depende The t1/2 was 4 min at 37 degrees C and 16 h at 6 degrees C. The association rate constant, k1, was also temperature-dependent, but not to the same extent k-1. 2. Plots of the observed rate constant for [3H]-QMDP-receptor complex formation, kon, versus [3H-QMDP] were linear at both 30 degrees C and 10 degrees C, consistent with the interaction of [3H]-QMDP with the H1-recept being a simple, one-step equilibrium. 3. The ratio of the kinetic constants, k1 1, indicated that the affinity constant of [3H]-QMDP for the H1-receptor sho increase with decreasing temperature. Measurement of (+)-QMDP antagonis of the contraction of longitudinal muscle strips from guinea-pig small intesti induced by histamine at 37 degrees C, 30 degrees C and 25 degrees C provid some evidence that the affinity of (+)-QMDP is greater at 25 degrees C than degrees C. However, the flattening of the concentration-response curves for histamine at low concentrations of (+)-QMDP at 30 degrees C and 25 degree C is consistent with a slow dissociation of the (+)-QMDP-receptor complex a hence an incomplete equilibration with the agonist. 4. Arrhenius plots for k1 and k-1 for [3H]-QMDP were linear between 37 degrees C and 6 degrees C. The activation energies, Ea, for complex formation and dissociation were 77 +/- 4 and 129 +/- 3 kJ mol-1, respectively. 5. An Arrhenius plot for k-1 for th dissociation of [3H]-mepyramine from the H1-receptor was also linear betwe 37 degrees C and 6 degrees C. The activation energy was 140 +/- 2 kJ mol-1. Activation energies for complex formation with the H1-receptor, Eaf, and complex dissociation, Ead, were similar for [3H]-QMDP and [3H]-mepyramine. The energy difference, Eaf--Ead, equivalent to the enthalpy change, did not differ significantly for the two ligands (-52 and -48 kJ mol-1, respectively). The larger values of k1 and k-1 for [3H]-mepyramine compare to [3H]-QMDP imply the presence of an entropic component in the interactic 7. The simplest explanation for these observations is that transfer from the

-37-

aqueous phase into a hydrophobic region is a significant factor in antagonist-H1-receptor interaction. This would be entropically more favourable for [3H mepyramine, a tertiary amine, than for [3H]-QMDP, a quaternary amine.

PMID: 3179614 [PubMed - indexed for MEDLINE]

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1: Eur J Pharmacol. 1986 Sep 9;128(3):231-9. Related Articles, Li

Evaluation of 4-methylpiperidine analogs of hemicholinium-3.

Tedford CE, Reed D, Bhattacharyya B, Bhalla P, Cannon JG, Long JP.

A series of substituted piperidine analogs of hemicholinium-3 was evaluated for their ability to inhibit neuromuscular transmission, to decrease acetylchol content of caudate slices, to inhibit choline acetyltransferase activity, and to produce toxicity. Quaternary and tertiary amine derivatives of 4-methyl- and hydroxyl-substituted piperidine analogs containing beta-carbonyl or beta-hydroxyl substitutions in the phenylethyl spacing moiety were tested. 4-Meth piperidine derivatives maintained potent hemicholinium-3 like activity. Reduction of activity was seen with the 4-hydroxyl piperidine analogs. Compounds with beta-hydroxyl substitution were more potent than those wit beta-carbonyl substitution. The tertiary amine, 4-methyl piperidine derivative with a hydroxyl group on the beta-carbon of the ethyl side chain also possess hemicholinium-3 like activity. However, tertiary amine analogs were substantially less potent than hemicholinium-3 or their quaternary amine analogs.

PMID: 3024994 [PubMed - indexed for MEDLINE]

☐ 1: Proc Natl Acad Sci U S A. 1976 Feb;73(2):452-6. Related Articles, Li

Amphipathic amines affect membrane excitability in paramecium: role for bilayer couple.

Browning JL, Nelson DL.

Amphipathic amines and local anesthetics stimulated reversal of the ciliary beating direction in wild-type Paramecium. Ca++ influx across the surface membrane and the consequent increase in internal Ca++ causes ciliary revers and backward swimming. Mutant cells of the "Pawn" class, which lack a "gating" mechanism for regulating Ca++ influx, did not swim backwards in t presence of local anesthetics. Local anesthetics stimulated the passive efflux K+ but had no effect on the active transport of K+ or Ca++. Apparently passi influx of Ca++ also was stimulated by local anesthetics as evidenced by their effects on swimming direction. These data can be interpreted in terms of the "bilayer couple" hypothesis of Sheetz and Singer [(1974) Proc. Nat. Acad. Sc USA 71, 4457-4461]: amphipathic drugs affect cells by asymmetric insertion into one face of the lipid bilayer. As predicted by this hypothesis, the drugs' effects were seen only after a short time lag, and quaternary amines were less effective than tertiary amines. The effect on behavior was caused by any of several amphipathic cations, and the relative potency was a function of their hydrophobicity. Amphipathic anions, which according to the hypothesis wou insert into the opposite face of the lipid bilayer, had little effect on ciliary reversal. Asymmetric perturbation of the lipid bilayer with amphipathic catio may trigger the opening of the Ca++ gate.

PMID: 1061147 [PubMed - indexed for MEDLINE]

1: J Pharmacol Exp Ther. 1978 Sep;206(3):595-606. Related Articles, Li

Carrier-mediated transport of the organic cation procaine amid ethobromide by isolated rat liver parenchymal cells.

Eaton DL, Klaassen CD.

Using hepatocytes isolated by collagenase perfusion, we studied the kinetic characteristics of the uptake process for procaine amide ethobromide (PAEB Determination of initial uptake velocities (Vo) at substrate concentrations fro 30 to 400 micrometer demonstrated a saturable process with a Km of 54 +/- micrometer and a Vmax of 0.13 +/- 0.01 nmol/min/mg of protein. Pretreatme of cells with metabolic inhibitors and reduction of the incubation temperatur significantly reduced the Vo of 100 micrometer PAEB. Replacement of sodi ions with lithium had no effect, while replacement with choline decreased V by 75%. The intracellular concentration of PAEB was 18 times the medium concentration after 90 min, but 33% of that was in the acetylated form. Uptal of N4-acetyl PAEB occurred at a much lower rate and reached a cell/mediur ratio of only 6 after 90 min. Only one of seven quaternary amines tested inhibited PAEB uptake at an inhibitor/substrate ratio (I/S) of 7.5, while four of five tertiary amines significantly decreased Vo at an I/S of 0.75 and all fiv decreased it at a ratio of 7.5. Some organic acids and steroidal compounds al significantly decreased PAEB Vo at an I/S of 0.75, while others from each group had no effect at an I/S of 7.5. Because uptake is saturable, requires metabolic energy, and occurs against an electrochemical gradient, it is suggested that the hepatic accumulation of PAEB occurs via an active, carrie medicated transport process.

PMID: 702324 [PubMed - indexed for MEDLINE]

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1: Arch Anat Histol Embryol. 1979;62:29-44. Related Articles, Li

[On the plurifactorial determinism of the organophosphorous-induced teratogenesis on bird embryos; trials of protection by various compounds: oximes, hydroxamic acids and nicotinamid analogs (author's transl)]

[Article in French]

Meiniel R, Quan DQ, Autissier-Navarro C, Caujolle R, Bernadou J.

Simple methods were applied to study the teratogenesis in Quail embryos induced by two important organophosphorous compounds: parathion and dicrotophos. Parathion led only to vertebral malformations, as other natural a synthetic cholinomimetics: nicotine, carbamylcholine, decamethonium, neostigmine... Dicrotophos induced not only vertebral malformations (specif to neuromuscular junction poisons) but also beak, legs and feather abnormalities (peripheric malformations which are also produced by insuline and sulfanilamide). Oximes and hydroxamic acids, some of these being analc of nicotinamide, were tested as antiteratogens. The 3-(CO-NH2), or -(CO-NHOH), substituted pyridinic compounds (nicotinamide, nicotinohydroxami acid) prevent perfectly dicrotophos-induced beak and legs malformations, in tertiary amine form, but very little in quaternary amine form (methyliodide). The 4-substituted pyridinic compound (isonicotinohydroxamic acid) and aliphatic oxo-oximes were quite ineffecient against these malformations. The vertebral malformations, as a rule, were not lessened by the compounds teste except for isonicotinoyl-formaldoxime methyl iodide and in some degree for nicotinohydroxamic acid. From these observations, it results that teratogenes induced by compounds as dicrotophos is rule by a plurificatorial determinism The beak and legs malformations are prevented by analogs of nicotinamide. I the contrary, the vertebral malformations induced by parathion or dicrotopho are nicotinamide unsensitive and are only prevented by powerful cholinester reactivators as pralidoxime or TMB4 (MEINIEL, 1976 b) but are reduced litt or not at all by less potent cholinesterase reactivators (HEATH).

PMID: 161493 [PubMed - indexed for MEDLINE]

☐ 1: Tohoku J Exp Med. 1982 May;137(1):91-9. Related Articles, Li

Effects of amine substances on susceptibility of cells to agglutination by concanavalin A and on paracrystal formation b vinblastine in untransformed 3T3 cells.

Saito K, Kumagai K.

The effects of a secondary amine (ketamine), tertiary amines (dibucaine, lidocaine, marcaine, propanidid, diazepam and chlorpromazine) and a quaternary amine (tetraethylammonium bromide, TEA) on mouse 3T3 cell agglutination by concanavalin A (Con A), on patch formation of Con A receptors on the cell surface, and on paracrystal formation by vinblastine in cytoplasm were studied. These amines enhanced the cell agglutination at low concentrations of Con A, as did the mixture of colchicine and cytochalasin B Ca++, applied extracellularly, inhibited the effects of these amines on cell agglutination by Con A. The patch formation of Con A receptors on the cell surface as revealed by fluoresceinated Con A was enhanced by these amines. Ketamine, dibucaine and TEA inhibited the paracrystal formation in cytoplas as did Ca++ ionophores such as A-23187 and X-537-A. These results sugges that the amines tested affect the fluidity of Con A receptors by impairment o cell membrane structural proteins and result in the increase of the susceptibil of cells to agglutination by Con A.

PMID: 6808705 [PubMed - indexed for MEDLINE]

1: Brain Res. 1990 Feb 12;509(1):41-6. Related Articles, Li

The effect of acetylcholine depletion on behavior following traumatic brain injury.

Robinson SE, Martin RM, Davis TR, Gyenes CA, Ryland JE, Enters EK

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond 23298.

Rats were injected with either saline; A-4 (40 mg/kg, i.p.), a bis tertiary amin derivative of hemicholinium-3; or A-5 (50 micrograms/kg, i.p.), a bis quaternary amine derivative of hemicholinium-3, 1 h prior to moderate fluid percussion brain injury. A variety of reflexes and responses were measured u to 60 min following injury, and body weight and several neurological measu were taken daily up to 10 days following injury. Pretreatment with either A-4 or A-5 significantly attenuated components of transient behavioral suppressi as well as more enduring deficits in body weight and beam walk and beam balance performance. A-4 administered prior to fluid percussion was found t reduce striatal, but not pontine, acetylcholine content. A-5 did not significant reduce acetylcholine content in either area. Both A-4 and A-5 pretreatment prevented a significant increase in acetylcholine content in the cerebrospinal fluid following fluid percussion injury; however, only A-5 significantly reduced plasma acetylcholine content. These results confirm cholinergic involvement in the production of both transient and longer-lasting behavioral deficits following traumatic brain injury. Furthermore, traumatic brain injury may allow plasma constituents to gain access to the central nervous system.

PMID: 2306637 [PubMed - indexed for MEDLINE]

1: Biull Eksp Biol Med. 1992 Mar;113(3):248-50. Related Articles, Li

[Use-dependent inhibition of C-axon multimodal units of cat ski by lidocaine and N-propyl-ajmaline]

[Article in Russian]

Mangusheva NA, Baidakova LV, Revenko SV.

Subcutaneous application of local anesthetic drug lidocaine and cardiac antiarrhythmic n-propyl-ajmaline produced the reversible use-dependent inhibition of feline polymodal mechano-heat C-fiber cutaneous sensory units (CMH-units) excited by moderate noxious mechanical stimulus. The dischar rate as well as the number of evoked spikes of polymodal sensory units treat with the drugs decreased below the values observed under noxious chemical excitation of CMH-units. The repeated mechano-stimulation with 5 to 30 sec interval between stimuli produced complete though a reversible block of the treated units. Quaternary amine n-propyl-ajmaline induced use-dependent inhibition of CMH-units in lower concentrations than tertiary amine lidocain The use-dependent inhibition of CMH-units is discussed in connection with nociception and local analgesia.

PMID: 1421216 [PubMed - indexed for MEDLINE]

1: J Pharm Biomed Anal. 1996 Aug;14(11):1425-33. Related Articles, Li

Effect of purification followed by solubilization of receptor material on quantitative receptor assays for anticholinergic dru;

Smisterova J, Ensing K, de Zeeuw RA.

Groningen Institute for Drug Studies, University Centre for Pharmacy, The Netherlands.

In order to optimize quantitative receptor assays for anticholinergics, the different receptor preparations resulting from the purification and the solubilization of the P2 pellet from the calf striatum were evaluated. The dissociation constants for two chemically different anticholinergics, the tertiε amine scopolamine and the quaternary amine oxyphenonium, were calculate from inhibition studies of 3H-NMS binding in buffer and plasma. The Kd values for both anticholinergics were similar for all the membrane-bound receptor preparations (unpurified and the purified P2 pellet) either in buffer c in plasma. More pronounced differences were observed between the membrane-bound and solubilized receptors. By introducing the solubilized receptor as well, differences between the individual anticholinergics appeare On the one hand, for scopolamine, a gain in sensitivity of 1.5-2.8 in plasma v observed for the solubilized receptor. On the other hand, in the case of oxyphenonium, a dramatic loss in sensitivity (by a factor of about 24) was observed with the solubilized receptor, as compared to the membrane-bound receptor, in buffer. Very interestingly, however, when the solubilized recepto was used in plasma, a lowering of the Kd value was found for both anticholinergics, i.e. the assays became more sensitive. Such an effect (not observed for the membrane-bound receptor) could be obtained only when the percentage of digitonin present in the assay was at least 0.12% (w/v) or high

PMID: 8877848 [PubMed - indexed for MEDLINE]

1: Biochim Biophys Acta. 1997 Oct 23;1329(2):211-22. Related Articles, Li

Erratum in:

- Biochem Biophys Acta 1998 Jun 24;1372(1):151.

Electrostatic parameters of cationic liposomes commonly used f gene delivery as determined by 4-heptadecyl-7-hydroxycoumari

Zuidam NJ, Barenholz Y.

Department of Biochemistry, The Hebrew University, Hadassah Medical School, Jerusalem, Israel.

Cationic liposomes are used to deliver genes into cells in vitro and in vivo. T present study is aimed to characterize the electrostatic parameters of cationic large unilamellar vesicles, 110 +/- 20 nm in size, composed of DOTAP/DOP (mole ratio 1/1), DOTAP/DOPC (mole ratio 1/1), 100% DOTAP, DMRIE/DOPE 1/1, or DC-CHOL/DOPE (mole ratio 1/1). inverted question markAbbreviations: DOTAP, N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide; DC-CHOL, 3beta[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol inverted question mark. The cationic liposomes had a large positive surface potential and a high pH at the liposomal surface in 20 mM Hepes buffer (pH 7.4) as monitored by the pH-sensitive fluorophore 4-heptadecyl-7-hydroxycoumarin. In contrast to DOTA and DMRIE which were 100% charged, DC-CHOL in DC-CHOL/DOPE (1/ liposomes was only about 50% charged in 20 mM Hepes buffer (pH 7.4). Th might result in an easier dissociation of bilayers containing DC-CHOL from plasmid DNA (which is necessary to enable transcription), in a decrease of tl charge on the external surfaces of the liposomes or DNA-lipid complexes, an in an increase in release of the DNA-lipid complex into the cytosol from the endosomes. Other electrostatic characteristics found were that the primary amine group of DOPE in cationic liposomes dissociated at high (> 7.9) pHbu and that a salt bridge was likely between the quaternary amine of DOTAP or DMRIE and the phosphate group of DOPE or DOPC, but not between the tertiary amine of DC-CHOL and the phosphate group of DOPE. The liposom containing DOTAP were unstable upon dilution, probably due to the high critical aggregation concentration of DOTAP, 7 X 10(-5) M. This might also

a mechanism of the dissociation of bilayers containing DOTAP from the plasmid DNA.

PMID: 9371413 [PubMed - indexed for MEDLINE]

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1: J Pharm Sci. 1999 Sep;88(9):922-7. Related Articles, Li

WILEY InterScience

A novel prodrug approach for tertiary amines. 2. Physicochemi(and in vitro enzymatic evaluation of selected N-phosphonooxymethyl prodrugs.

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Quaternary amine prodrugs resulting from N-phosphonooxymethyl derivatization of the tertiary amine functionality of drugs represents a novel approach for improving their water solubility. Separate reports have demonstrated the synthetic feasibility and rapid and quantitative prodrug to parent drug conversion in rats and dogs. This work is a preliminary evaluatio of the physicochemical and in vitro enzymatic reversion properties of selecte prodrugs. The loxapine prodrug had over a 15 000-fold increase in aqueous solubility relative to loxapine free base at pH 7.4. The loxapine prodrug was also shown to be quite stable at neutral pH values. The time for degradation product (parent drug) precipitation from an aqueous prodrug formulation wo be expected to dictate the shelf life. Using this assumption, together with solubility and elevated temperature chemical stability studies, the shelf life o parenteral formulation of the loxapine prodrug was projected to be close to 2 years at pH 7.4 and 25 degrees C. In addition, the prodrugs of cinnarizine an loxapine have been shown to be substrates for alkaline phosphatase, an enzy found throughout the human body, and revert to the parent compound in its presence. The results from these evaluations demonstrate that the derivatives examined have many of the ideal properties required for potential clinical application.

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